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CONSECUTIVE NO. 14

A Synthesis of Diketopiperazines Using Polyphosphoric Acid*

By ALVIN M. GALINSKY, JAMES E. GEARIEN, and EDWARD E. SMISSMAN†

Conversion of an *alpha*-amino acid to its corresponding 2,5-diketopiperazine was accomplished by heating the amino acid with a suitable dehydrating agent such as polyphosphoric acid. This procedure, after the excess polyphosphoric acid was decomposed and neutralized, when applied to glycine, alanine, leucine, isoleucine, and phenylalanine, gave good yields of the corresponding diketopiperazines. The temperature of the reaction is the most critical factor in obtaining satisfactory yields. The optimum reaction conditions for each of the five amino acids are reported.

A METHOD IS presented for the conversion of *alpha*-amino acids to diketopiperazines. Glycine, alanine, leucine, isoleucine, and phenylalanine were converted to their corresponding diketopiperazines by the action of polyphosphoric acid. Cysteine and methionine were decomposed by the reagent.

Interest in substituted 2,5-diketopiperazines as intermediates in the synthesis of organic medicinals has been increased by their conversion into substituted piperazines (1) and into substituted 2,5-dichloro-3,6-dihydropyrazines (2). At present there exists no satisfactory general method for the direct conversion of alpha-amino acids to their corresponding diketopiperazines. Most published syntheses of these compounds require the conversion of the amino acid ester to the diketopiperazine with ammonia (3, 4) or the cyclization of a dipeptide ester under similar conditions (5).

Synthesis of substituted diketopiperazines directly from the amino acid would eliminate the necessity of preparing intermediates and might offer an improved method of preparation. Sannié (6) prepared 2,5-diketopiperazine by heating glycine with large amounts of ethylene glycol. Kohler (7) obtained 3,6-diisobutyl-2,5-diketopiperazine by heating dry leucine in a stream of dry hydrogen chloride. While Sannié's method was applicable to several amino acids, he was unable to obtain yields exceeding fifty per cent. It appeared that the yield of substituted 2,5-diketopiperazine might be increased if the amino acid was heated in a suitable dehydrating agent such as polyphosphoric acid. When such a procedure was followed and the excess polyphosphoric acid decomposed and neutralized, glycine, alanine, leucine, isoleucine, and phenylalanine all gave good yields of the corresponding diketopiperazines. Cysteine and methionine, however, were decomposed during heating with polyphosphoric acid.

The effects of reaction time, temperature, and the ratio of amino acid to polyphosphoric acid were studied. It appeared that the temperature of the reaction was the most important factor in obtaining satisfactory yields. Too low tempera-

* Received January 22, 1957 from the Department of Chemistry, College of Pharmacy, University of Illinois, Chicago 12.

The authors wish to thank the Victor Chemical Works for the generous supplies of polyphosphoric acid.

† Present address: School of Pharmacy, University of Wisconsin, Madison.

This paper is based on a thesis submitted by Alvin M. Galinsky to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the degree of Master of Science.

TABLE I.—INFLUENCE OF TEMPERATURE ON THE YIELD OF DIKETOPIPERAZINE

Starting Amino Acid	Yield, %, of Corresponding Diketopiperazine at Reaction Temperatures of—					
	70–75°	75–80°	80–85°	85–90°	90–95°	95–100°
Glycine	0	20.6	55.0	63.2	63.2	42.5
Alanine	...	15.0	53.8	53.8	33.7	...
Leucine	70.6	52.1	0	...	...	...
Isoleucine	0	...	...	0	25.7	62.4
Phenylalanine	0	...	0	38.2	74.8	60.8

NOTE: All other factors, except temperature, were fixed for maximum yield.

tures resulted in the isolation of unreacted amino acid while too high temperatures caused decomposition of the product and reactants. The effect of temperature upon yield is shown in Table I.

While the time necessary for the conversion of the amino acid to substituted diketopiperazine varied with each amino acid, it was found that the maximum yield was obtained when the reaction mixture was heated for thirty minutes after complete solution of the amino acid. It was found that 200 cc. of polyphosphoric acid was a convenient volume with which to work. The maximum amounts of amino acid that could be converted to the corresponding diketopiperazine are stated in the experimental section.

Attempts to isolate the diketopiperazines by neutralization of the diluted reaction mixture with sodium or potassium hydroxide were unsuccessful due to the large amounts of inorganic phosphates formed. Only 3,6-diisobutyl-2,5-diketopiperazine could be isolated by this procedure. To eliminate the formation of large amounts of inorganic phosphates, the neutralization was carried out with an ion exchange resin. For the more water-soluble substituted 2,5-diketopiperazines, the diluted reaction mixture was neutralized by passage through a column containing the resin. The more water-insoluble substituted 2,5-diketopiperazines, however, precipitated out on the resin. These were best isolated by neutralizing with the resin in a batch procedure to a pH of 5 and, after removal of the resin, to a pH of 7 with sodium hydroxide.

Commercially available polyphosphoric acid was found to be as effective in the reaction as that which was prepared extemporaneously in the laboratory (8).

The method does not appear to be applicable to the preparation of optically active substituted 2,5-diketopiperazines. When *l*-leucine was converted to 3,6-diisobutyl-2,5-diketopiperazine an optically inactive product was obtained.

EXPERIMENTAL

General Procedure for the Preparation of 2,5-Diketopiperazine and Substituted 2,5-Diketopiperazines.—A mixture of 10–20 Gm. of the amino acid, 200 cc. of polyphosphoric acid, and 1.0 Gm. of phosphorus pentoxide was heated at 70–100° with constant stirring for one and one-half to six hours. The resulting solution was then poured into a beaker containing 200 Gm. of cracked ice and stirred until the excess polyphosphoric acid was decomposed. A clear solution resulted. Sufficient water was added to bring the total volume of the solution to 1500 cc. and the solution was neutralized to a pH of 7.

Neutralization for the more water-soluble substituted diketopiperazines was carried out by passing the diluted reaction mixture through a column two inches in diameter which had been filled with Amberlite IR 4B (OH) ion exchange resin to a height of 18 inches. It was necessary to pass the solution through the regenerated resin a second time to obtain complete neutralization. The column was washed with distilled water to insure complete removal of the organic material from the column.

When the solubility of the diketopiperazine in water was not sufficiently high for use of the column, a "batch" process was employed. The diluted reaction mixture was stirred in a large beaker with Amberlite IR 4B (OH) ion exchange resin until the resin was exhausted. The resin was then filtered off, washed with distilled water and the washings added to the partially neutralized solution. This process was repeated until a pH of 5 had been reached. Final neutralization to a pH of 7 was completed with 20% sodium hydroxide solution.

When the solubility data indicated, the solution was cooled in an ice bath to induce crystallization. Otherwise the solution was evaporated at 35° in a flash-evaporator to approximately 125 cc. The resulting substituted 2,5-diketopiperazines were purified by recrystallization from the proper solvents.

2,5-Diketopiperazine.—Fifteen grams (0.2 mole) of glycine was heated with 200 cc. of polyphosphoric acid and 1.0 Gm. of phosphorus pentoxide for three hours at 85–90° with constant stirring. The excess polyphosphoric acid was decomposed with ice water and the resulting solution neutralized with ion exchange resin by the column procedure. After evaporation, the neutral solution yielded a solid which was recrystallized from water. The yield was 7.2 Gm. (63.2%) of a white solid which sublimed at 256–257° and decomposed at 308–310°. Fischer (3) reported that 2,5-diketopiperazine sublimed at 256° and decomposed at 310°.

3,6-Dimethyl-2,5-diketopiperazine.—A mixture of 10.0 Gm. (0.11 mole) of *dl*-alanine, 200 cc. of polyphosphoric acid and 1.0 Gm. of phosphorus pentoxide was heated with constant stirring, at 80–90° for four hours. The product was isolated in a manner identical with that employed in the synthesis of 2,5-diketopiperazine. After recrystallization from eth-

anol, the yield was 4.2 Gm. (54%). The white crystalline solid melted at 272–274°, reported m. . . 271° (9).

3,6- Diisobutyl - 2,5 - diketopiperazine.—Nineteen and seven-tenths Gm. (0.15 mole) of *l*-leucine was added to 200 cc. of polyphosphoric acid containing 1.0 Gm. of phosphorus pentoxide. This mixture was heated, with constant stirring at 70–75° for one and one-half hours. The reaction mixture after dilution with ice water was neutralized to pH 7 with sodium hydroxide solution. The flocculent precipitate, which formed immediately, was filtered off and recrystallized from 70% ethanol. The yield was 12.0 Gm. (71%) of a white crystalline solid which melted at 269–271°, reported m. p. 271° (3). A solution of the compound in glacial acetic acid did not rotate a plane of polarized light.

3,6-Di-sec-butyl-2,5-diketopiperazine.—A mixture of 19.7 Gm. (0.15 mole) of *dl*-isoleucine and 200 cc. of polyphosphoric acid was heated for three and one-half hours at 95–100° with constant stirring. The excess polyphosphoric acid was decomposed with cracked ice and neturalized with ion exchange resin and sodium hydroxide by the "batch" process. After cooling, a solid precipitated which after recrystallizaiton from 50% ethanol, yielded 10.6 Gm. (62%) of a white cr stalline solid melting at 280–282°, reported 280–281° (4).

3,6-Dibenzyl-2,5-diketopiperazine.—Sixteen and one-half grams (0.10 mole) of *dl*-phenylalanine was added to a mixture of 200 cc. of polyphosphoric acid and 1.0 Gm. of phosphorus pentoxide. The mixture was heated at 90–95° for four hours with constant stirring. The excess polyphosphoric acid was then decomposed with ice water and neutralized with ion exchange resin and sodium hydroxide using the "batch" procedure. After partial evaporation of the water, a white solid separated. Upon recrystallization from ethanol, 11.0 Gm. (75%) of the compound melting at 283–285°, reported 290–291° (10), was obtained.

Anal. Calcd. for $C_{18}H_{18}O_2N_2$: N, 9.52%. Found N, 9.58%.

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A Pharmacognostical Study of *Datura discolor* Bernh*

By PAUL H. KALEMKIARIAN and ORVILLE H. MILLER

***Datura discolor* is commonly found in the Colorado desert of Southern California and in parts of Mexico. It is described in several keys to flora of this area, but has never been examined pharmacognostically. The historical background, distribution, and growth habits of this plant are discussed. A taxonomic description is included and covers additional features not presented in previous reports. The principal microscopic characteristics are described and compared with other *Datura* species of this area. Alkaloidal content and composition of a number of samples are reported. Total alkaloids approximate those of the official *Datura stramonium*, but the mixture contains a much greater proportion of scopolamine.**

THERE are a number of native and naturalized *Datura* species found in the United States such as *Datura stramonium*, *Datura tatula*, *Datura inermis*, *Datura meteloides*, and *Datura discolor*. All of these species except *Datura discolor* have been investigated for their alkaloidal content and have been occasionally utilized for drug purposes.

Datura discolor is a native plant found in the southern part of California, Arizona, and New Mexico as well as Mexico. It has frequently been enumerated in floras covering these regions (1–7). *Datura discolor* was first described by Bernhardi in 1833 (8). In 1856 Torrey (9) described a *Datura Thomasii* which was later assumed to be the same species (10, 11). *Datura discolor* is quite common in the Colorado desert area of southeastern California and in Mexico, particularly in areas bordering the Gulf of California. *Datura meteloides* is often found in these same areas and may be confused with it, although they can be readily differentiated as will be shown later. Both species are usually found in desert washes and along roadsides where there has been a disturbance of the soil.

PHARMACOGNOSTICAL STUDY

A study of 115 *Datura discolor* specimens from herbarium collections aided in determining the distri-

* Received April 17, 1956, from the School of Pharmacy, University of Southern California, Los Angeles. Abstracted in part from a dissertation presented to the Graduate School of the University of Southern California by Paul H. Kalemkiarian in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacy.
Presented to the Scientific Section, A. PH. A., New York meeting, April-May, 1957.

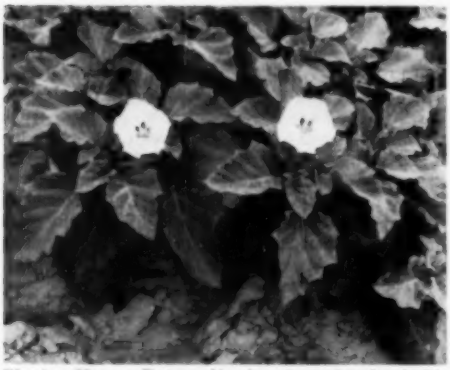

Fig. 1.—Young *Datura discolor* plant showing habit and flower with five purple flushes in corolla.

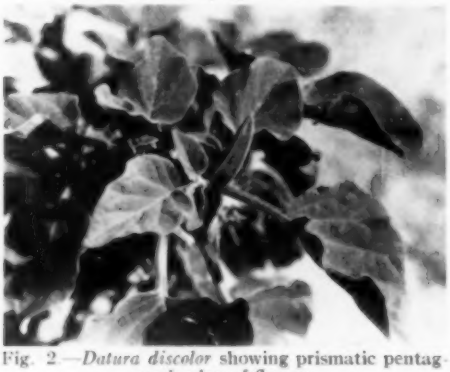

Fig. 2.—*Datura discolor* showing prismatic pentagonal calyx of flower.

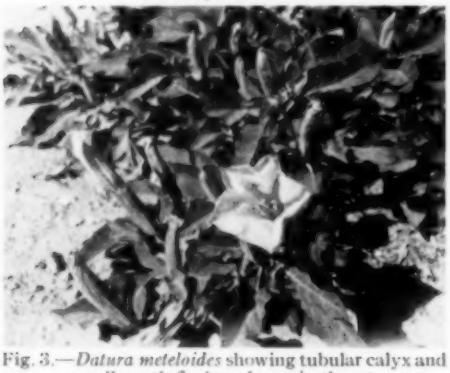

Fig. 3.—*Datura meteloides* showing tubular calyx and corolla with flushes absent in throat.

bution of the specie. Specimens had been collected in Arizona, California, New Mexico, and Mexico in the states of Lower California, Sonora, Sinaloa, Nayarit, Jalisco, Guerroro, Oaxaca, Chihuahua, San Luis Potasi, Colima, and Tamaulipas. The collection dates for these specimens ranged from 1859 through 1947.

Specimens for field studies and for experimental work were obtained from eastern Southern California and adjoining areas in Mexico. Thirteen different specimens were studied and collected. Most collections were made during January and February of

Fig. 4.—A—capsule fruit of *Datura discolor* showing sturdy spines; B—capsule fruit of Datura meteloides showing small spines.

1950 and 1951. Plant material was most abundant following periods of rainfall in the desert areas where the specie thrives. Specimens were observed and collected at the following localities: Indio, Palo Verde, Blythe, Desert Center, Niland, and Dos Palmas Ranch in California, and near Mexicali and San Felipe in Mexico.

The taxonomic descriptions of *Datura discolor* were found to be incomplete in a number of respects for pharmacognostical purposes. Therefore the following description was developed from field studies.

Formal Description of Datura Discolor.—Annual herb, 1 to 3 feet tall; cinerious, pubescent; tap roots 8 to 12 inches long, 1/2 to 3/4 inch in diameter, rootlets numerous, slender; stems erect, diffuse, forking, internodes hollow; leaves petiolate, blades broadly ovate, sinuate-dentate, usually 1 to 4 1/2 inches long, but may be as much as 9 inches long, petioles usually 1 to 4 inches long; calyx prismatic, 1 1/2 to 2 inches long, terminating in 5 acute teeth at apex; corolla trumpet shaped, 4 or 5 inches long, white, with 5 slender subulate teeth alternating with 5 obtuse teeth at the rim and 5 purplish flushes inside the throat about 1/4 of an inch wide and 1/2 to 3/4 of an inch long; anthers white; capsule nodding, globose, tetralobular at base, bilocular at apex, 1 to 1 1/2 inches in diameter not including spines, dehiscing by four valves, spines pubescent, stout, 1/2 to 3/4 inch long, calyx collar reflexed; seeds black, rugulose, coarsely pitted, 3.5 to 3.9 mm. long by 2.6 to 3.0 mm. wide by 1.5 mm. thick with a small white aril.

Datura discolor can be readily differentiated from *Datura meteloides* in the field by the following characteristics:

	D. discolor	*D. meteloides*
Calyx	Prismatic, pentagonal	Tubular
Corolla	White with 5 purplish flushes inside throat	White to faintly colored. Flushes absent
Fruit	Large sturdy spines up to 3/4 in. long	Numerous small spines up to 3/8 in. long
Seeds	Black, reniform, coarsely pitted with small white aril	Light to medium brown, reniform flattened with wrinkled rim

Fig. 5.—A—seeds of *Datura meteloides;* B—seeds of *Datura discolor;* C—seeds of *Datura stramonium.*

Datura stramonium and *Datura tatula* can also be readily differentiated from *Datura discolor* in that their seeds while black are less pitted and without the white aril. Their leaves have a shiny upper surface relatively free of plant hairs, whereas they are abundant on *Datura discolor* producing a soft dull texture. Furthermore, the large sturdy spines on the fruit of *Datura discolor* readily differentiate it from all the other species apt to be associated with it. The differences described above can be noted in Figs. 1 to 5.

Microscopical Characteristics.—Two common major microscopical differences in the leaf drugs from the *Solanaceae* are in the types of calcium oxalate crystals present in the leaves, and the types of hairs present on the leaves. Differences in these characteristics were also found adequate for a microscopical differentiation of *Datura discolor* and *Datura meteloides.* A description of these characteristics follows:

(*a*) Calcium oxalate crystals. The calcium oxalate crystals in the leaf of *Datura discolor* as seen through its thickness, after clarification with chloral hydrate, were mainly in the form of prisms, occasionally twin prisms (as seen in *Hyoscyamus muticus* leaves), and a few clusters or rosette forms. The average size for these crystals was 10 to 25 microns (Fig. 6). *Datura meteloides* shows numerous rosettes and cluster crystals, very rarely a few prisms. The average diameter of these rosettes was 10 to 15 microns (Fig. 7).

(*b*) Hairs. *Datura discolor* leaves show numerous hairs on both surfaces, both glandular and nonglandular. The nonglandular hairs were multicellular, uniseriate trichomes, usually 3 to 5 cells high, and about 100 to 200 microns long, with a warty surface. The glandular hairs usually have a base 1 to 2 cells high, with a unicellular gland, or a multicellular gland usually with 2 to 8 cells. Average size of base is 30 to 50 microns long and gland 50 to 80 microns in diameter (Fig. 8). *Datura meteloides* leaves have very few hairs on the upper surface, all composed of nonglandular, multicellular, uniseriate trichomes, usually 2 to 4 cells high, and 130 to 260 microns long, with a warty surface. The lower surface shows numerous nonglandular trichromes, similar to the ones on the upper surface, and rarely a few glandular hairs with a unicellular or bicellular base, and a unicellular or bicellular gland.

The Alkaloids of Datura Discolor.—The crude drugs obtained from the *Solanaceae* family, yielding

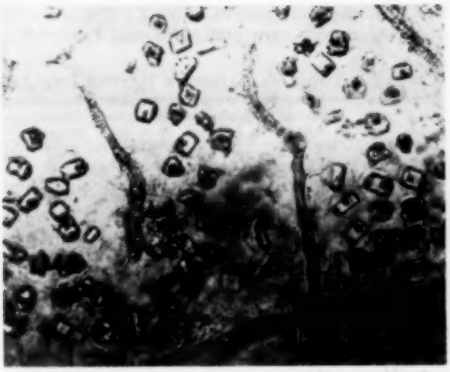

Fig. 6.—Calcium oxalate crystals of *Datura discolor.*

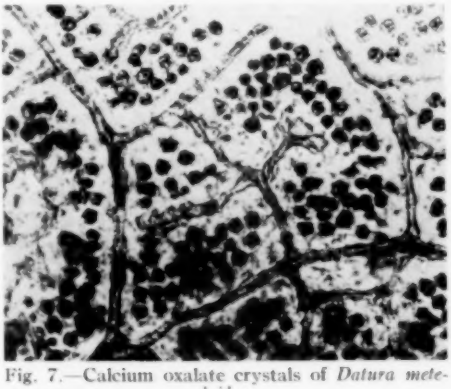

Fig. 7.—Calcium oxalate crystals of *Datura meteloides.*

Fig. 8.—Transverse section of *Datura discolor* leaf showing hairs on upper and lower surfaces.

the tropane alkaloids, can be assayed by the method prescribed in the United States Pharmacopœia, Fourteenth Revision (12). This method of assay for determining the amount of total alkaloids in belladonna is identical with the method used for Hyoscyamus and Stramonium in the National Formulary, Ninth Edition (13). The total alkaloids of *Datura discolor* were determined by this procedure

and the results appear in Table I. Two or more replicate assays were performed and the average

TABLE I.—TOTAL ALKALOIDS IN DATURA DISCOLOR

Specimen	Alkaloids in Whole Plant, %	Alkaloids in Leaves, %	Alkaloids in Stems, %	Alkaloids in Roots, %
13	0.31	...	...	...
14	...	0.36	0.17	0.16
15	...	0.40	0.33	...
38	...	0.44	0.36	0.32
39	...	0.30	0.36	0.13
40	...	0.20	0.14	...
41	...	0.27	0.22	...
42	0.17	...	...	...
43	0.30	...	...	...
44	0.34	...	...	...
45	...	0.19	...	...
46	...	0.36	0.16	...
47	...	0.49	0.19	...
Av.	0.28	0.33	0.21	0.20

reported. The samples of plant material had been previously collected and dried in a circulating oven at 50–60°, then ground to #40 powder.

The alkaloidal extract obtained in the assays was shown to contain tropane alkaloids by a positive Vitali test for tropane alkaloids. The presence of scopolamine was determined by the Kuhn and Schafer method (14), and approximated 50% of the total alkaloid. The identity of the scopolamine was further confirmed by the melting point of the aurichloride of the separated fraction. Melting point 205–206° corresponds to reported values of 208–209° (15). A turbidity was produced by gold chloride solution in the remaining fraction but no crystalline material was obtained, indicating this fraction to be a mixture of alkaloids. Further work is in progress on the fractionation of the alkaloidal constituents of *Datura discolor*.

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The Effect of Ceanothyn on Blood Coagulation Time*

By JACK G. BISHOP† and ALFRED W. RICHARDSON‡

Oral administration of 3.5–7.0 ml. Ceanothyn decreased blood coagulation time for a period of approximately 1 hour. Accelerated clotting was noted within 10 to 20 minutes after administration. Repeated oral doses of Ceanothyn kept the blood coagulation time diminished. Dialysed Ceanothyn reduced clotting time when added to whole blood, indicating that the active fraction was of small molecular size. At low concentrations Ceanothyn acted as a coagulant, while increasing the concentration caused it to become an anticoagulant.

HYDROALCOHOLIC EXTRACTS prepared from the root bark of *Ceanothus americanus* have been employed clinically as a coagulant for many years, under the name Ceanothyn,® according to Taylor (1). Groot (2) reported an average decrease of 30% in clotting time in response to doses of 1/4 to 1/2 ounce of Ceanothyn, and a 41.7% decrease in coagulation time in one group of 13 patients. He also found that coagulation time depression corresponded, within limits, to the amount of drug administered orally. Tharaldsen and Krawetz (3) confirmed these findings and reported that this action may take

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place independently of the action of digestive enzymes. Payne (4) cited 225 cases in which he used drug preoperatively to hasten coagulation and to control hemorrhage, and found a decrease of 21.9% in coagulation time.

Since the capillary tube method used by these workers for measuring coagulation time was quite variable, serious doubt has been cast upon these observations. The procedure for obtaining blood samples was not standardized, and insufficient controls were obtained for reliability. It was not clarified in these studies in what manner Ceanothyn acted in the clotting mechanism to achieve a coagulant effect.

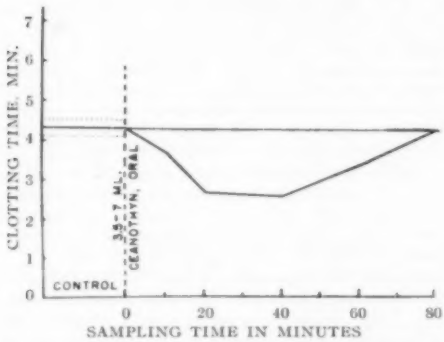

Fig. 1.—Decreased coagulation time in response to 3.5–7 ml. Ceanothyn administered orally. Standard error indicated by dotted line.

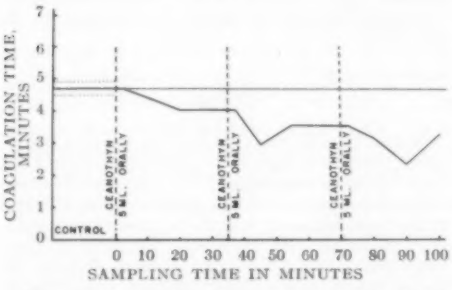

Fig. 2.—Diminution in coagulation time as shown in venous blood due to successive oral administration of 5 ml. Ceanothyn at 35-minute intervals.

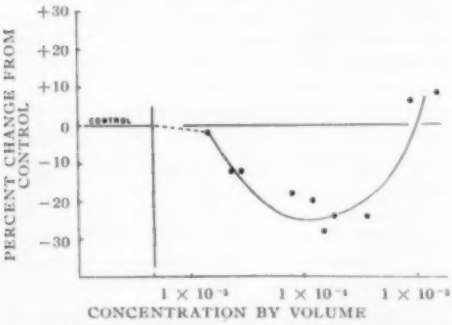

Fig. 3.—Per cent change from control by the addition of varying concentrations of Ceanothyn by volume.

PROCEDURE

To ascertain the effectiveness of Ceanothyn as a coagulant, 24 mongrel dogs, weighing 9 to 18.8 Kg. body weight, were used. The animals were anesthetized with sodium pentobarbital, 33 mg./Kg. This prevented undue excitation and allowed optimal reliability of the measurements.

Three and one-half to seven ml. of Ceanothyn was introduced into the stomachs of dogs by means of a stomach tube and washed in by saline. Samples of blood were removed before and after this procedure to serve as controls for the animal.

Blood-clotting time was determined by the recording method developed by Richardson and Bishop (5). Blood samples were obtained by venipuncture in a well-drained, paraffin oil-coated 5-ml. syringe, with a paraffin oil-coated 22-gauge needle. The Esterline-Angus record was started as blood entered the syringe. The needle was removed from the syringe and one ml. of blood was carefully discharged into the polystyrene blood container. The electrodes were inserted in a horizontal position and rotation started immediately. Clotting time was determined on dual samples obtained by a single venipuncture. Samples from the first two venipunctures and the last two venipunctures served as controls for the animal.

Figure 1 shows the average results obtained from 9 dogs: 25 control samples and 39 experimental samples. Twenty minutes post-Ceanothyn there was a 26% reduction in coagulation time. This level was maintained for twenty minutes and returned to control level within 80 minutes after the administration of Ceanothyn.

Figure 2 shows the effect of successive doses of Ceanothyn orally upon coagulation time. Control samples were obtained by venipuncture previous to the administration of Ceanothyn. Five to ten ml. of Ceanothyn was introduced, by stomach tube, and

TABLE I.—RESULTS OF DIFFERENT CONCENTRATION OF CEANOTHYN ON BLOOD COAGULATION TIME

Condition	No.	Dilution Ratio	Concentration by Volume	Coagulation Time, Min.
Controls	88			5.0
Ceanothyn	96			
	8	1:53,500	1.87×10^{-5}	4.9
	10	1:26,750	3.74×10^{-5}	4.4
	10	1:21,400	4.67×10^{-5}	4.4
	24	1:10,700	9.34×10^{-5}	4.1
	6	1:9,000	1.12×10^{-4}	4.0
	8	1:5,350	1.87×10^{-4}	3.6
	6	1:3,570	2.80×10^{-4}	3.8
	4	1:1,785	5.60×10^{-4}	3.8
	7	1:1,070	9.34×10^{-4}	5.3
	5	1:535	1.87×10^{-3}	5.4
	8	1:100	1.00×10^{-2}	No clot in 15 min.

samples were taken at approximately 10-minute intervals. A second dose of the same amount as previously used was given approximately 35 minutes after the initial dose, and this was followed by a third dose 70 minutes after the initial dose. The control-clotting time was 4.7 ± 0.3 minutes. This dropped to 4.0 after the first Ceanothyn dose and was maintained at this level for 35 minutes. The second dose of Ceanothyn decreased the clotting time to 2.9, with a recovery to 3.5 at the end of 70 minutes after the first administration. The third dose of Ceanothyn decreased the coagulation time to 2.5, but required longer to do so, whereupon the clotting time slowly returned to control level. No toxic effect was observed.

TABLE II.—EFFECT OF DIALYSED CEANOTHYN ON COAGULATION TIME

Condition	Proportion of Ceanothyn to Blood	No. of Samples	Coagulation Time, Min.
Controls (6 dogs)		49	4.7
Dialysed Ceanothyn	1:80	11	4.4
	1:40	8	3.5
	1:20	12	3.3

A group of experiments was designed to assay the effect of Ceanothyn on blood coagulation when diluted each side of the estimated range obtained from *in vivo* studies. Dilutions of Ceanothyn were added to freshly drawn blood from anesthetized dogs, and the coagulation time determined on 88 controls and 96 experimental samples. In this series, graded concentrations of Ceanothyn were added to whole blood, the concentrations varying progressively from 1 part Ceanothyn to 53,500 parts blood; 1 part Ceanothyn to 100 parts blood. Greater concentrations of Ceanothyn were used and found to inhibit coagulation. The results of these measurements are revealed in Table I and Fig. 3, where it can be seen that with the progressive addition of Ceanothyn in these very low concentrations, the coagulation time of blood is progressively diminished to a minimum value; further addition of Ceanothyn reverses the curve, as shown by gradual prolongation of coagulation time.

TABLE III.—THE EFFECT OF CEANOTHYN-CALCIUM CHLORIDE MIXTURES ON COAGULATION TIME

Conditions	Mixture Dilution in Blood	No. of Samples	Coagulation Time, Min.
Controls (12 dogs)	...	65	5.3
Ceanothyn + $CaCl_2$ (1:1.04 mg./ml.)	1:100	10	5.3
Ceanothyn + $CaCl_2$ (1:1.5mg./ml.)	1:100	6	3.5
Ceanothyn + $CaCl_2$ (1:10 mg./ml.)	1:100	11	4.6
Ceanothyn + $CaCl_2$ (1:20 mg./ml.)	1:100	14	4.9
Ceanothyn + $CaCl_2$ (1:22.2 mg./ml.)	1:100	6	4.9
Ceanothyn + $CaCl_2$ (1:40 mg./ml.)	1:100	17	5.0

Two millimeters of Ceanothyn was carefully introduced into a collodion sac and immersed in 20 ml. of saline for 12 hours to ascertain whether the active principle was a substance or substances of small molecular size which would pass through a dialysing membrane. The dialysate plus saline was added to fresh blood in proportions of 1:80, 1:40, and 1:20, and the results are shown in Table II. These findings suggest that at least one active component of Ceanothyn is dialysable, and comparing these values with those of diluted Ceanothyn, it is apparent that only a portion of the active substance was separated.

In an effort to further clarify the mechanism by which Ceanothyn effects coagulation of blood, a series of experiments was designed in which graded concentrations of calcium were added to Ceanothyn and this mixture added to whole blood. A total of 12 animals was used in these studies and the average control coagulation time was 5.3 minutes. Results are shown in Table III. When calcium chloride and Ceanothyn were mixed immediately before adding to blood, coagulation was inhibited, except in concentrations of 1% calcium. By mixing the samples several hours before use, and then adding the mixture to whole blood, coagulation occurred. This procedure was employed for these data. One part of Ceanothyn by volume to 1 part of calcium chloride (1.04 mg./ml.) apparently had no effect on coagulation time. Increasing the concentration of calcium to 1.51 mg./ml. resulted in a decreased coagulation time of 34%. Further increase of this amount of calcium chloride to 1% decreased the clotting time 13.2%, while a 4% calcium chloride-Ceanothyn mixture almost returned clotting to control level.

COMMENT

These findings indicate that by the removal of small amounts of calcium from the blood, Ceanothyn may act as a coagulant. The roots of *Ceanothus americanus* contain sodium oxalate, Wirth (6), and resins have been reported to be present in Ceanothyn, Groot (2) and Clark (7). Withdrawal of calcium ions from the blood upsets the equilibrium between bound and unbound calcium, according to Norbdo (8), Martin (9), and frees the prothrombin for subsequent conversion into thrombin. The prothrombin-to-thrombin reaction was facilitated by the presence of calcium ions; therefore, if sufficient quantities of Ceanothyn were added to remove the calcium ions sufficiently, delayed clotting or no clotting was the result.

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Sulfaethylthiadiazole I.*

Interpretations of Human Blood Level Concentrations Following Oral Doses.

By JOSEPH V. SWINTOSKY, MANFORD J. ROBINSON, ELWOOD L. FOLTZ,† and SPENCER M. FREE

Blood concentration studies were conducted for a ten or twelve hour period on eleven human subjects receiving sulfaethylthiadiazole orally in 1–4-Gm. doses. The drug disappearance rate from the blood was characterized as approximately first-order, from which the standard performance indices k_b, $(t^{1}/_{2})_b$ and C_o were determined. This approach to characterization of drug disappearance rates from the blood also permitted a close estimation of the per cent of total drug in the blood which existed in the biotransformed state. For this type of drug it is suggested that the methods of analysis presented can furnish useful scientific criteria for better evaluation of drug performance, and that in industrial pharmacy it can guide the pharmaceutical project team in formulation and establishment of dosage schedules.

IN THE STUDY of some chemotherapeutic agents, measurements of the concentrations obtained in the blood after administration provide data of value in determining time-dose relationships (1). Such data are particularly useful in the design of dosage forms and regimens when the rate of disappearance of drugs from the blood can be defined mathematically and suitable constants are obtained which describe the drug performance. For a number of drug and nondrug substances, plots of blood plasma concentrations against time have been shown to decrease approximately exponentially for a considerable period after establishment of absorption and "pseudo steady state" diffusion equilibrium. This has been illustrated for such substances as streptomycin (1), caffeine (2), sulfathiazole (3), creatinine (4), and others. Teorell (10, 11), Dominguez (4), and Lapp (12) have given detailed mathematical treatment to the kinetics of absorption, distribution, and disappearance of ingested substances.

Since it has been shown that several sulfonamides disappear from the blood stream at approximately first-order rates (3), a study was initiated with sulfaethylthiadiazole (SETD), a drug of current medical interest. This study was conducted to determine the blood concentration picture of this drug when administered orally, in various doses, to human subjects. Specifically, it was desired to quantitate the disappearance and biotransformation processes for the drug in the blood.

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Presented to the A.A.A.S., New York meeting, December, 1956.

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EXPERIMENTAL

Protocol for Human Studies.—Eleven healthy young adult white male human subjects were used in this study, several at more than one dosage level. For some subjects the SETD was administered in 0.5-Gm. hard gelatin capsules in oral doses of 1, 2, 3, and 4 Gm. and blood samples were withdrawn at 0, 1, 2, 3, 4, 6, 8, and 10 hours. Other subjects received capsules in oral doses of 1, 3, and 4 Gm., or 4 Gm. in aqueous suspension and had blood samples withdrawn by venipuncture of the antecubital veins at 0, 1, 2, 4, 6, 8, and 12 hours. Administration was in the morning, to subjects in the fasting state, and control blood samples were taken before administration of the medication. Breakfast followed drug ingestion within an hour. Subjects were ambulatory, and after their breakfast were permitted to ingest food and fluids as desired.

Analytical Methods.—Concentrations of free and free plus conjugated (total) SETD in the blood were determined by a modification of the macromethod described by Frisk (5). Spectrophotometric measurements of the final colored solutions were made with a Beckman Spectrophotometer, Model B, at a wavelength of 550 mμ. Comparisons were made with appropriate standards for dilutions and origin of samples.

RESULTS AND DISCUSSION

Typical blood concentrations obtained with a 2-Gm. oral dose of SETD are shown in Table I. For most of the test subjects, where oral doses ranged from 1 to 4 Gm., peak blood concentrations occurred in from 1 to 3 hours.

Drug losses from the blood of an individual frequently may be characterized, after termination of absorption and attainment of diffusion equilibrium, by the first-order rate expression (3).

$$\log C = -k_b t/2.303 + \log C_o \qquad (1)$$

The blood concentration data of this study were plotted according to this straight line equation. Relatively high blood concentrations were attained for the doses administered. For subjects showing

TABLE I.—FREE AND FREE PLUS CONJUGATED (TOTAL) SETD BLOOD CONCENTRATIONS FOR FOUR SUBJECTS RECEIVING 2-GM. ORAL DOSES IN HARD GELATIN CAPSULES

	Blood Concentrations in mg./100 cc.							
	Subject, *Ro*		Subject, *Bs*		Subject, *Cw*		Subject, *Ds*	
Hour[a]	Free	Total	Free	Total	Free	Total	Free	Total
1	9.6	10.0	10.2	10.4	11.2	11.4	10.8	11.2
2	7.9	8.0	8.5	8.6	9.8	9.8	11.2	11.4
3	8.0	7.8	7.8	8.0	8.6	9.0	10.5	11.0
4	7.6	7.6	7.0	7.3	7.8	8.6	9.7	10.0
6	6.0	6.3	5.3	5.5	6.9	7.3	8.7	9.0
8	5.0	5.4	4.8	4.8	5.7	6.2	8.8	8.6
10	4.3	4.5	4.0	4.2	4.8	5.5	7.3	7.4

[a] Control blood samples obtained just prior to drug administration indicated zero concentration of drug.

peak blood concentrations between 1–3 hours, the data of succeeding hours plotted quite satisfactorily as straight lines on semilog scale graph paper, similar to previously reported plots (6) for intravenously administered SETD. This is illustrated for subject *Cw* at several doses in Fig. 1. The consistency of these findings in all subjects was sufficient to suggest that, under the conditions of this study, disappearance of SETD from the human blood following 1–4-Gm. oral doses, may be approximated conveniently as a first-order rate. The extent to which this is true for subjects of more variable health, age, etc., remains to be established.

Previously (1, 3), it has been inferred that for the interpretation of a considerable body of drug blood concentration data, a useful approach utilizes the standard performance indices k_b, $(t_{1/2})_b$ and C_o. In routine work it has been found rather impractical to employ rigorous mathematics in order to obtain the numerical values of these indices. Where sufficient data are available, and the analytical method for obtaining them is precise enough, the position of the straight lines on semilog paper may be determined quite accurately by inspection. By this method, individual values of $(t_{1/2})_b$ and C_o can be determined quickly, directly from the graphs. The values of each k_b are then determined from the relationship

$$k_b = 0.693/(t_{1/2})_b \quad (2)$$

and, average k_b =
0.693/Harmonic mean of $(t_{1/2})_b$ values (3)

In this initial report on the standard performance indices for orally administered SETD it seemed desirable to employ the more rigorous mathematical approach. The methods of Boxer, *et al.* (1), have been helpful in the tabular presentation of the data. The k_b values for each subject at each dose were determined by analyzing each set of data according to Eq. 1, by the method of least squares. The combined data of Fig. 1 illustrate that in the determination of the standard performance indices, each set of oral blood concentration data must be considered separately because of the differences in drug absorption rates. In this study, the two-hour blood concentration data were used as the first points on the linear response curves for most subjects. The three-hour data were used for the remaining subjects. One set of data was discarded because the linear response could not be readily defined. This apparently was due to a slow and very prolonged absorption time.

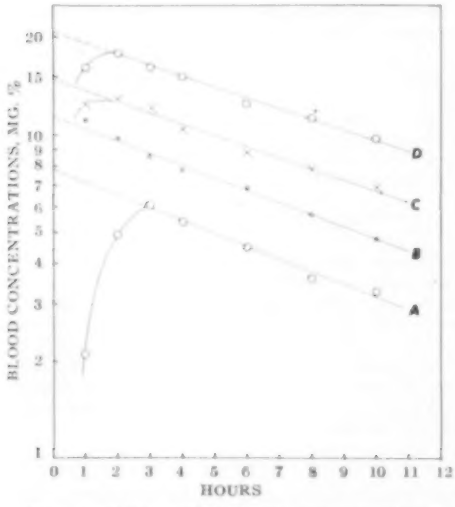

Fig. 1.—Sulfaethylthiadiazole concentrations in the blood for subject *Cw* following oral doses of (A) 1.0, (B) 2.0, (C) 3.0, and (D) 4.0 Gm.

DOSE RANGE STUDIES

The average k_b of Eq. 3 is the arithmetic mean of the individual values of k_b. This method of expressing the average as a standard performance index assumes that the individually determined values for this index are not influenced by the C_o values within the dose range studied. The data shown in Table II indicate that this is a fairly good assumption; and that drug disappearance rate from the blood in these subjects was relatively unaffected by the size of the dose. Thus, within a practical dose range, the average k_b and its variability appear to be satisfactory for expressing drug performance; and can serve as a useful guide to the research pharmacist, pharmacologist, clinician, and project team in product formulation and evaluation. Similarly, the harmonic mean $(t_{1/2})_b$, an alternate index related to k_b, can serve the same useful purpose.

If one desires to place limits about $(t_{1/2})_b$, following the assumptions outlined above, it is necessary to find an expression for the variability of k_b, since the individual $(t_{1/2})_b$ values do not conform to the usual statistical requirements for normal distribution. This is commonly done by a procedure known as Feiller's Theorem (7) where fiducial limits are determined.

It is well known that the sulfonamides now in clinical use undergo some biotransformation in the body. The important type of biotransformation consists of acetylation at the N^4-amino group or similar conjugation. Nevertheless the biotransformed products are readily detectable and measurable; and can be determined quantitatively in body fluids or tissues. Thus, another index of drug performance of SETD is per cent biotransformed drug in the tissues.

The per cent biotransformed drug in the blood (% B_b) of each individual at any time covered by this study as determined from blood concentration data, may be given as

$$\% B_b = (\text{Total drug} - \text{free drug})/\text{total drug} \times 100 \quad (4)$$

If, as the data of Tables I and II imply, one considers the rate of drug disappearance from the blood to be nearly the same for free and total SETD at the concentrations studied, the estimation of biotransformed drug in the blood can be approached as a biological assay with parallel line responses. The perpendicular distances, *d*, between the lines for the free and total drug response would be due to biotransformation. Mathematically, the estimation of this biotransformation is readily accomplished by making both lines parallel, determining the C_o for each line and solving the equation

$$\% B_b = [1 - (C_o \text{ free drug})/(C_o \text{ total drug})] \times 100 \quad (5)$$

The parallel line method for estimating per cent biotransformed drug in the blood was applied to the data obtained in this study. This method is illustrated in Fig. 2 for the data of subject *Cw* at a 4-Gm. dose. Following the assumptions outlined for averaging the individual values of k_b, one can justify an arithmetic average for an estimate of biotransformation in all the subjects and doses of this study.

Results of the assumptions and calculations as applied to the experimental blood concentration data are illustrated in Figs. 1 and 2 and Table II. The ± values indicate standard errors of the mean. Inspection of the k_b values for free drug indicate a range of 4.7–12.9%/hour, corresponding to a $(t_{1/2})_b$ range of 5.4–14.4 hours. For total SETD data, k_b varied from 5.2–12.6%/hour, corresponding to a $(t_{1/2})_b$ range of 5.5–13.3 hours. Average k_b values for free and total SETD were 8.4 ± 0.4 and 8.3 ± 0.4%/hour, respectively. Harmonic mean values for $(t_{1/2})_b$ were 8.2 and 8.4 hours, with fiducial limits of 7.4–9.2 and 7.5–9.4 hours, respectively, for free and total drug. As indicated, the variations in the k_b values for free and total drug lie within the standard error of the mean values. Therefore, disappearance rates of free and total drug may be considered identical.

The data suggest that under the conditions of this study, k_b and $(t_{1/2})_b$ are independent of the dose and whether capsules or suspensions were administered. The values of C_o are dose dependent, but are fairly consistent at each dosage level, considering variations in subject weights and absorption rates. At the 1-Gm. dose the geometric mean C_o is 6.9 mg. %. This value is of considerable theoretical interest, as it bears on calculations of steady state SETD blood concentrations following a sequence of 1-Gm. doses at regular time intervals, a sequence commonly followed in the dosage regimens of sulfonamide therapy.

Table II indicates that the amount of biotransformed drug in the blood is very low, ranging from about 1–14% of total drug, with an average of about 5%. This is a very low order of biotransformation, and corroborates the blood concentration and urinary excretion studies of Frisk (5).

The per cent of conjugated drug in the human blood is lower with SETD than with most other orally administered sulfonamides (5, 8, 9). The extremely small biotransformation undergone by this compound is a desirable attribute, since the N^4-conjugated sulfonamides lack therapeutic efficacy and are sometimes troublesome by virtue of their insolubility and their tendency to produce crystalluria. The possibility of such complication in humans due to biotransformation of SETD would appear to be ruled out on the basis of our findings and those previously reported (5). The small amount of conjugation which occurs after absorption of SETD enhances therapeutic efficacy and is a valuable quality of this drug.

It is believed that the determination of the standard performance indices, as illustrated in this study, may be especially useful in evaluating this sulfonamide, and also other drugs where there is good correlation between drug tissue concentration and therapeutic efficacy. Where applicable in connection with pharmaceutical product development, this approach to blood concentration studies, following oral doses, appears to lend itself to more critical scientific interpretation than the usual tabular data or Cartesian coordinate plots. The usefulness of such standard performance indices in describing and interpreting blood concentration-time curves for some drugs has assumed greater importance in pharmacy as greater responsibilities befall the pharmacist in incorporating special features

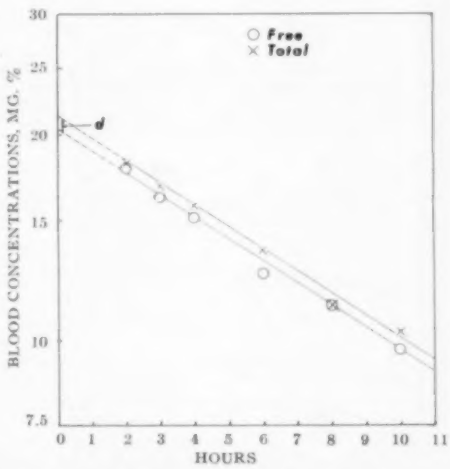

Fig. 2.—Sulfaethylthiadiazole blood concentrations for subject *Cw* at 4-Gm. dose, illustrating parallel line method for estimating per cent biotransformed drug in the blood.

TABLE II.—STANDARD PERFORMANCE INDEXES FROM BLOOD CONCENTRATION DATA FOLLOWING ORAL ADMINISTRATION OF SETD

Subject	Wt., Kg.	Age Yr.	Sex	Mode of Administration	Dose (Gm.)	C_o (mg. %)	Free Drug k_b (%/hr.)	$(t_{1/2})_b$ (hr.)	C_o (mg. %)	Total Drug k_b (%/hr.)	$(t_{1/2})_b$ (hr.)	B_b, %
Ro	91	22	M	Capsules	1.0	5.1	6.7	10.3	5.3	6.6	10.5	4.1
Bs	114	38	M	Capsules	1.0	4.9	8.6	8.1	5.0	7.6	9.1	7.4
Cw	91	22	M	Capsules	1.0	7.8	9.0	7.7	7.9	9.0	7.7	1.2
Ds	81	25	M	Capsules	1.0	9.1	7.2	9.7	9.2	6.9	10.1	1.9
Dy	89	34	M	Capsules	1.0	6.2	7.6	9.1	6.6	7.8	8.9	5.2
Da	79	26	M	Capsules	1.0	7.2	9.6	7.2	7.1	8.4	8.2	4.9
Rm	64	22	M	Capsules	1.0	8.9	10.9	6.3	10.0	12.6	5.5	1.4
Ss	75	27	M	Capsules	1.0	7.3	11.6	6.0	7.9	12.2	5.7	4.0
Ro	"	"	M	Capsules	2.0	10.0	8.2	8.4	9.7	7.2	9.6	2.9
Bs	"	"	M	Capsules	2.0	10.2	9.6	7.2	10.4	9.4	7.4	2.7
Cw	"	"	M	Capsules	2.0	11.3	8.6	8.1	11.3	7.3	9.5	6.8
Ds	"	"	M	Capsules	2.0	12.1	4.8	14.4	12.6	5.2	13.3	2.0
Ro	"	"	M	Capsules	3.0	12.9	7.7	9.0	13.8	7.8	8.8	5.7
Bs	"	"	M	Capsules	3.0	11.6	8.9	7.8	12.3	9.2	7.5	4.0
Cw	"	"	M	Capsules	3.0	14.8	7.9	8.8	15.0	7.6	9.1	3.1
Ro	"	"	M	Capsules	4.0	16.0	6.2	11.2	16.8	6.3	11.1	4.3
Bs	"	"	M	Capsules	4.0	15.3	8.8	7.9	16.0	9.1	7.7	2.9
Cw	"	"	M	Capsules	4.0	20.4	7.5	9.3	21.0	7.3	9.5	3.5
Dc	"	"	M	Capsules	4.0	18.1	6.0	11.5	18.9	6.1	11.4	3.8
Ck	160	28	M	Suspension	4.0	18.6	12.9	5.4	20.0	11.7	5.9	13.9
Ms	77	26	M	Suspension	4.0	18.4	5.7	12.1	19.3	5.4	12.8	6.1
Rm	"	"	M	Suspension	4.0	16.6	7.6	9.2	18.1	7.8	8.9	7.1
Zn	79	23	M	Suspension	4.0	24.3	12.3	5.6	24.7	11.7	5.9	5.7
Average[b]	...	...	...	...	...	...	8.4 ± 0.4	8.2	...	8.3 ± 0.4	8.4	4.6 ± 0.1

[a] As given in preceding Figures.
[b] Data for $(t_{1/2})$ are harmonic mean values.

in dosage forms which regulate drug release, absorption, distribution, and excretion. They have considerable theoretical and practical utility in evaluating drug performance, and in guiding the pharmaceutical project teams of industry in formulation and establishment of dosage schedules.

SUMMARY

1. Sulfaethylthiadiazole was administered orally to 11 young adult male human subjects and drug blood concentrations were determined for 10–12 hours following administration.

2. Absorption of the drug was rapid, often giving peak blood concentrations within 1–2 hours following 1–4-Gm. oral doses. Two hours after a 2-Gm. oral dose, blood concentrations of free drug ranged from 8 to 11 mg. per cent.

3. Blood concentrations of free or total drug followed a first-order disappearance rate after the attainment of peak concentrations.

4. The methods of this study permitted a determination of standard performance indices which included k_b, $(t_{1/2})_b$, C_o, and per cent biotransformed drug in the tissues.

5. For these subjects the average k_b equalled 8.5 per cent/hour and $(t_{1/2})_b$ equalled 8 hours (to the closest 0.5 per cent/hour and hour, respectively) for free SETD. It was demonstrated that the indices for total SETD were essentially identical to those determined for free SETD.

6. Taking into consideration varying weight and absorption rates, C_o values corresponded rather closely for each subject at single dosage levels.

7. The average amount of drug in the tissues in the biotransformed state was about 5 per cent, an amount consider-

ably lower than that observed in the case of most other currently available sulfonamides.

8. This approach to blood concentration studies, in connection with pharmaceutical product development, appears to lend itself to critical scientific interpretation. Specifically, it makes possible better evaluation of drug performance and may be especially helpful in guiding the scientific and clinical personnel of pharmaceutical product development teams in formulating drug products and devising their dosage schedules.

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Sulfaethylthiadiazole II.*

Distribution and Disappearance from the Tissues Following Intravenous Injection

By JOSEPH V. SWINTOSKY, MANFORD J. ROBINSON, and ELWOOD L. FOLTZ†

Sulfaethylthiadiazole was readily compounded as an injectable aqueous solution, and was administered intravenously without difficulty to four healthy adult human subjects in doses ranging from 0.5–2 Gm. High blood concentrations were attained. Elimination rates obtained from blood concentration and urinary excretion data were determined to be approximately first-order for a considerable portion of a dose, and the rates obtained from both blood and urine tests corresponded closely. These data were also employed to determine the apparent distribution volume of the drug. The results of the study suggest that this sulfonamide may be administered conveniently by the intravenous route, and the compound has a utility in investigations of drug absorption, distribution, excretion, kidney function, and time-dose studies.

IN PREVIOUS PUBLICATIONS (1, 2, 3, 13) it has been shown that at therapeutic levels, sulfaethylthiadiazole (SETD) and several other sulfonamides exhibit approximately first-order disappearance rates from the tissues following oral or intravenous doses. From preliminary data (2, 3) the rate equations, approximating the elimination process for SETD, appear to be followed rather well over considerable tissue concentrations of drug. The ability to administer a drug intravenously and to approximate its disappearance rate from the tissues is of academic and practical importance in studies of clinical efficacy, absorption, and excretion (4, 5, 6, 9, 12). It is often of practical importance in the pharmaceutical industry, especially in correlating dosage form design with suitable dosage regimens (1).

With orally administered SETD (2) it was assumed that the beginning of the straight line semilog paper plots of blood concentrations *versus* time corresponded to the time after which the absorption from the gastrointestinal tract had virtually ceased, and renal elimination primarily accounted for drug disappearance from the blood. Data can be obtained bearing on the credibility of this assumption by administering the drug intravenously in doses of such magnitude that concentrations in the blood

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may be determined with considerable accuracy. If and when, after the elapse of some time, the semilog paper blood concentration *versus* time plots of orally administered drug have the same slopes as intravenous plots, this may denote the time for the virtual completion of the oral absorption process (1).

This study of drug disappearance rate from the blood following intravenous injections of SETD, therefore, was conducted to determine the ease and safety with which the drug could be administered intravenously, and to relate disappearance rates of drug administered intravenously to rates reported previously for orally administered doses. Other objectives of the study were to determine, (*a*) the time required for venous SETD to attain "pseudo steady state" diffusion equilibrium with other body fluids, (*b*) how drug disappearance rate from the tissues is influenced by doses varying between 0.5 and 2 Gm., (*c*) the degree to which disappearance rate varies between individuals, (*d*) the apparent distribution volume and apparent per cent distribution volume, (*e*) the degree to which these volumes vary between individuals at different doses, (*f*) the relationship after diffusion equilibrium between blood concentrations and total drug in the body, and (*g*) the extent of biotransformation of SETD.

Analyses of the observed results and data were performed to determine the utility of urinary excretion data in establishing SETD disappearance rate from the tissues, and to determine the interplay of factors relating drug disappearance from the blood to urinary excretion rate.

EXPERIMENTAL

Solution for Injection.—The formula of the solution was as follows:

Sulfaethylthiadiazole (American Cyanamid)	25.0 Gm.
Diethanolamine (Carbide and Carbon)	9.5 cc.
Distilled water, *q. s.*	100.0 cc.

The solution was filtered through a fine porosity glass sintered filter, and autoclaved in 10-cc. ampuls. The pH of the solution was 7.5–8.1.

Protocol for Human Studies.—Four healthy ambulatory male subjects were employed. The drug was administered in doses of 0.5, 1, 1.5, and 2 Gm. into the antecubital veins. The 0.5 and 1-Gm. doses were diluted with normal saline solution, in a syringe, to a volume of 10 cc., and administered in approximately 6 minutes. The 1.5 and 2.0-Gm. doses were diluted up to 30 cc. and administered in 10–12 minutes. The SETD was administered in the morning to subjects reporting in a fasting state, and after a control blood sample had been collected. Breakfast followed the $^1/_2$-hour collection of the first blood sample. Other blood samples were collected at 1, 2, 4, 6, and 8 hours. The collections of blood for sulfonamide concentration were made by venipuncture of the antecubital veins.

The subjects were urged to take a minimum of 200 cc. of water by mouth at 0, 3, 6, 9, 12, and 15 hours following SETD administration. After the morning meal, food was ingested as desired. Collections of total urinary output were made at 3, 6, 9, 12, 15, 24, and 48 hours for the 0.5- and 1-Gm. doses. Some collections up to 72 hours were made for the 1.5- and 2-Gm. doses. Volumes were measured and recorded. Aliquots were retained for analyses of concentrations and drug outputs.

Analytical Methods.—Concentrations of free as as well as free and conjugated (total), SETD in the blood and urine were determined by a modification of the macromethod described by Frisk (7). Spectrophotometric observations of the final colored solutions were made with a Beckman Spectrophotometer, Model B, at a wavelength of 550 mμ. Comparisons with appropriate standards for dilutions and origin of samples were made for blood and urine samples.

RESULTS AND DISCUSSION

Blood Concentrations.—No problems were encountered in administering this drug intravenously. The experimental blood concentration and urinary excretion data are presented in Tables I and II. From Table I it may be noted that the free and total SETD blood concentrations are very nearly identical; and it is evident that biotransformed drug in the blood averages about 4%. This value is essentially identical with that observed following oral administration of SETD (2).

The free SETD blood concentration data of Table I are plotted for a single subject in Fig. 1. The curves decrease exponentially for all doses during the 2–8-hour interval. The exponential relationship also is demonstrable if total drug blood concentrations *versus* time are plotted, and essentially equivalent slopes are obtained for free and total drug. The data of Fig. 1 are representative for all subjects. The slopes of the straight line plots correspond closely to those observed for orally (2) administered SETD. The deviations from the straight lines in the very early time intervals are interpreted as variations due to nonequilibrium diffusion of drug between blood and other fluid compartments of the body. This equilibrium appears to be established after approximately one hour. It is interesting that the various doses require approximately the same time to attain diffusion equilibrium. This would be expected if the mixing and distribution are also subject to the laws governing processes which decrease exponentially.

The specific velocity constant, k_b, and biologic half-life, $(t_{1/2})_b$, for SETD disappearance from the blood at the times and concentrations studied are readily determined graphically, or analytically, by the use of the first-order rate equation defined previously (1). The values for these constants as determined from the 2–8-hour data are presented in Table III. The experimental values for $(t_{1/2})_b$ vary from 5 to 9.5 hours, with an harmonic mean of about 7.5 hours. This compares favorably with the range of 5 to 14 hours and harmonic mean of 8 hours observed in a larger group of subjects given the drug orally (2). This amount of variation is believed to be quite normal and is consistent with similar observations on other drugs (1). The harmonic mean values of $(t_{1/2})_b$ and average k_b for each

TABLE I—BLOOD CONCENTRATION DATA FOR HUMAN SUBJECTS RECEIVING INTRAVENOUS SETD

		Blood Concentrations in mg. %; and % Conjugated Drug (% Conj.)											
		0.5 Gm.			1 Gm.			1.5 Gm.			2 Gm.		
Subject	Hour	Free	Total	% Conj.	Free	Total	% Conj.	Free	Total	% Conj.	Free	Total	% Conj.
Ro	½	3.8	3.8	0	6.6	6.7	2	9.5	9.5	0	10.9	11.5	6
	1	3.2	3.5	9	5.5	5.5	0	7.5	7.6	1	9.1	9.4	3
	2	2.4	2.7	11	4.3	4.7	8	6.4	6.6	3	7.7	8.2	6
	4	2.1	2.1	0	3.6	3.8	5	5.2	5.3	2	5.7	6.1	7
	6	1.8	1.8	0	3.0	3.0	0	4.5	4.6	2	4.8	5.2	8
	8	1.5	1.6	6	2.5	2.5	0	3.9	4.1	5	3.0	3.0	0
Bs	½	3.3	3.3	0	5.9	5.9	0	6.9	7.1	3	8.8	9.1	3
	1	2.5	2.5	0	5.2	5.2	0	6.3	6.4	2	7.9	8.0	1
	2	2.1	2.2	5	4.4	4.4	0	4.8	5.5	13	6.6	6.6	0
	4	1.9	1.9	0	3.8	3.9	3	4.4	4.7	6	5.4	5.4	0
	6	1.5	1.6	6	3.0	3.2	6	3.8	3.9	3	4.2	4.2	0
	8	1.4	1.5	7	2.5	2.7	7	3.1	3.4	9	3.6	3.8	5
Cw	½	3.8	4.0	5	7.6	7.7	1	9.7	10.0	3	11.6	12.8	9
	1	3.0	3.2	6	6.8	6.8	0	8.2	8.8	7	10.7	10.9	2
	2	2.5	2.8	11	5.6	5.6	0	7.2	7.4	3	9.4	9.4	0
	4	2.1	2.5	16	4.5	4.6	2	5.8	6.1	5	7.2	8.0	10
	6	1.8	2.0	10	3.6	3.6	0	5.0	5.3	6	6.2	6.8	9
	8	1.4	1.5	7	3.1	3.4	9	4.8	4.8	0	5.2	5.6	7
Ds	½	4.3	4.4	2	9.6	10.3	7	11.8	11.8	0	13.6	14.2	4
	1	3.8	3.8	0	8.6	8.6	0	9.6	10.3	7	12.0	12.4	3
	2	3.3	3.3	0	7.6	7.6	0	8.3	8.5	2	10.2	11.0	7
	4	2.9	2.9	0	5.9	6.1	3	7.2	7.3	1	8.5	9.1	7
	6	2.4	2.4	0	4.9	4.9	0	6.3	6.3	0	7.2	7.4	3
	8	1.9	2.0	10	4.0	4.1	2	5.4	5.8	7	5.8	6.2	6

individual at the various doses are fairly constant despite a considerable weight variation, and despite minimal regulation of fluid and food intake during the course of the investigation.

It is of considerable theoretical and practical interest that the straight line first-order rate equation plots of drug disappearance from the blood are essentially identical following intravenous administration or oral administration of the drug in capsules or liquid suspensions (2). The slopes of the straight line plots as reported previously for 1–4-Gm. oral doses (2) generally correspond, after 1–3 hours, to the slopes obtained after intravenous administration. For oral doses, these straight line plots began at or shortly after the appearance of peak blood concentrations. This is interpreted to mean that for oral doses the absorption was usually terminated in the 1–3-hour interval following administration. Therefore, for this drug, the technique of administration via the intravenous and oral routes, coupled with semilog paper plots of the respective blood concentration data, appears to permit a close examination of the period, and time of cessation, of principal absorption following an oral dose (1).

Urinary Excretion.—The experimental urinary excretion data are presented in Table II. Free and total SETD excreted in the various time intervals correspond closely. Experimental determinations of the urine collected from these four individuals indicate that generally about 70–90%, 85–100%, and 90–100% of the dose is excreted via this route in 24, 48, and 72 hours, respectively. These results are quite similar to observations of Frisk (7). After an intravenous dose of this drug essentially 100% may be accounted for in the urine, with an average of 8% in the conjugated form. Though this value is slightly higher than that found for per cent biotransformed SETD in the blood (2), it confirms the fact that biotransformation is of a very low order.

Since essentially all of the injected drug is excreted in 72 hours, one may utilize this 72-hour figure to represent total excretable drug. The amounts remaining in the body after each urine collection time are then readily computed. A plot of the log of unexcreted drug *versus* time follows an approximately first-order rate after attainment of diffusion equilibrium. Such elimination rate via the urinary tract is defined by the equations (1, 8).

$$\log C = (-k_u t/2.303) + \log C_o \qquad (1)$$

$$\text{and } (t_{1/2})_u = 0.693/k_u$$

The subscripts "*b*" and "*u*", as used in this paper, differentiate the standard performance indexes, respectively, according to whether they are derived from blood concentration or urinary data. In Fig. 2, the urinary excretion data are plotted for the four subjects receiving 1.5 Gm. SETD by the intravenous route. The values of C were computed with the assumption that total drug excreted in 72 hours represented total excretable drug. As noted previously for a 2-Gm. dose (3), after attainment of diffusion equilibrium, the approach to a first-order rate is fairly good through an extensive time interval, with a $(t_{1/2})_u$ ranging from 9 to 10.5 hours.

The disadvantages of this method of obtaining and plotting data are that it requires a long period of urine collection and that total excretable drug must be measured accurately. Also, errors in the measurement of C are cumulative with time, so that any slight consistent error in one direction could force the data out of line at a later time. Any deviation from the relationship defined by Eq. 1 is also magnified on semilog paper plots at the lowest excretion levels. Despite this, the data of Fig. 2 plot quite well with respect to rate, for each subject. The values of $(t_{1/2})_u$ are slightly higher than the values of $(t_{1/2})_b$ at the same doses.

A method for determining the excretion rate during the early time intervals which overcomes some

TABLE II.—URINARY EXCRETION DATA FOR HUMAN SUBJECTS RECEIVING INTRAVENOUS SETD

Subject	Collection Time Interval, Hr.	Urinary Volumes in cc. and Drug Excreted in mg., for Intervals Indicated											
		0.5-Gm. Dose			1.0-Gm. Dose			1.5-Gm. Dose			2.0-Gm. Dose		
		Urine Volume, cc.	Free, mg.	Free and Conjugated,[a] mg.	Urine Volume, cc.	Free, mg.	Free and Conjugated, mg.	Urine Volume, cc.	Free, mg.	Free and Conjugated, mg.	Urine Volume, cc.	Free, mg.	Free and Conjugated, mg.
Ro	0–3	225	29	29	290	296	290	222	435	477	645	800	855
	3–6	330	43	44	275	110	113	127	170	181	547	137	164
	6–9	300	80	80	205	115	115	153	168	207	690	207	235
	9–12	320	54	54	270	92	92	152	94	103	475	238	276
	12–15	300	38	41	170	63	63	149	92	101	248	104	124
	15–24	255	77	90	760	78	122	543	155	163	1,238	161	192
	24–48	860	80	82	3,140	94	107	2,700	227	257	1,200	64	70
	48–60	...	...	...	...	...	...	...	...	...	...	...	...
	60–72	...	...	...	...	...	...	...	...	...	...	...	...
	48–72	...	...	...	...	...	...	2,350	52	59	...	...	...
	Sum	...	401 (48 Hr.)	420 (48 Hr.)	...	848 (48 Hr.)	902 (48 Hr.)	...	1,393 (72 Hr.)	1,548 (72 Hr.)	...	1,711 (48 Hr.)	1,916 (48 Hr.)
Bs	0–3	445	107	107	170	204	230	101	364	394	253	577	620
	3–6	750	64	68	710	99	107	358	211	226	1,087	609	652
	6–9	285	27	29	710	96	103	273	117	120	268	204	204
	9–12	280	35	39	240	79	82	316	115	107	217	130	135
	12–15	700	42	44	265	58	68	255	92	101	645	142	152
	15–24	690	64	68	830	120	125	982	196	216	1,190	220	224
	24–48	1,425	80	83	1,555	114	132	1,730	190	208	2,393	170	191
	48–60	...	...	...	...	...	...	...	...	...	...	...	...
	60–72	...	...	...	...	...	...	...	...	...	...	...	...
	48–72	...	...	...	...	...	...	1,820	58	60	1,290	40	41
	Sum	...	409 (48 Hr.)	433 (48 Hr.)	...	770 (48 Hr.)	847 (48 Hr.)	...	1,343 (72 Hr.)	1,432 (72 Hr.)	...	2,092 (72 Hr.)	2,219 (72 Hr.)
Cw	0–3	225	113	113	200	204	225	1,445	441	477	1,170	562	644
	3–6	150	71	75	75	129	146	120	187	204	153	245	245
	6–9	930	47	48	105	92	102	93	97	107	114	192	211
	9–12	240	48	49	81	60	65	162	130	138	455	187	209
	12–15	945	50	51	75	44	47	95	72	86	1,280	154	160
	15–24	945	58	60	765	134	134	694	146	191	970	53	71
	24–48	1,480	44	44	1,160	128	148	820	172	230	1,272	178	223
	48–60	...	...	...	...	...	...	360	25	36	...	...	...
	60–72	...	...	...	...	...	...	1,146	23	29	...	...	...
	48–72	...	...	...	...	...	...	...	...	...	1,755	49	58
	Sum	...	431 (48 Hr.)	440 (48 Hr.)	...	791 (48 Hr.)	867 (48 Hr.)	...	1,293 (72 Hr.)	1,498 (72 Hr.)	...	1,620 (72 Hr.)	1,821 (72 Hr.)
Ds	0–3	107	91	94	120	269	286	520	437	468	445	534	590
	3–6	515	75	75	125	145	150	215	181	194	968	436	474
	6–9	1,220	77	79	130	83	94	140	143	147	252	181	197
	9–12	300	27	29	465	56	70	113	84	96	248	139	146
	12–15	100	20	22	530	74	87	89	78	82	276	110	132
	15–24	355	50	51	595	104	113	250	120	140	1,840	202	221
	24–48	690	64	67	1,000	125	145	515	118	162	796	195	207

TABLE II.—(*Continued*)

Ds	48–60	...	...	...	...	...	...	290	29	38	...	...	...	
	60–72	...	...	...	...	...	...	302	23	24	...	...	...	
	48–72	...	...	...	...	...	...	...	...	...	544	52	60	
	Sum	...	404 (48 Hr.)	417 (48 Hr.)	...	856 (48 Hr.)	945 (48 Hr.)	...	1,213 (72 Hr.)	1,351 (72 Hr.)	...	1,849 (72 Hr.)	2,027 (72 Hr.)	

[a] Free and conjugated SETD are expressed in mg. equivalents of SETD.

TABLE III.—DATA CALCULATED FROM BLOOD CONCENTRATION AND URINARY EXCRETION STUDIES ON HUMAN SUBJECTS RECEIVING INTRAVENOUS SETD

1 Subject	2 Height in.	3 Weight, Kg.	4 Age, Yr.	5 Dose, Gm.	6 $(t^1/_2)_b$[a] Hr.	7 k_b[b] %/Hr.	8 Interpolated Total SETD Blood Level at 3 Hr., mg. %	9 Total Drug In the Body at 3 Hr., mg.[c]	10 V_b, cc. From 3-Hr. Data	11 Total SETD Blood Level at 6 Hr., mg. %	12 Total Drug in the Body at 6 Hr., mg[c]	13 V_b, cc. From 6-Hr. Data	14 V_b, cc. Calculated Av. from Columns 10 & 13	V_b % Av. V_b/V_s	16 $(t^1/_2)_a$ (Hr.) Detd. from Eq. 1	17 k_a[b] (%/Hr.)
Ro	78	91	22	0.5	9	7.7	2.4	471	19,600	1.8	427	23,700			...	...
				1.0	8	8.7	4.2	710	16,900	3.0	597	19,900			...	...
				1.5	9.5	7.3	5.9	1,023	19,000	4.6	842	18,300			10.5	6.5
				2.0	5	13.9	7.2	1,145	15,900	5.2	981	18,900			...	...
													19,000	21		
Bs	72	114	38	0.5	8	8.7	2.0	393	19,600	1.6	325	20,300			...	...
				1.0	7	9.9	4.1	770	18,800	3.2	663	20,700			...	...
				1.5	8	8.7	5.1	1,106	21,700	3.9	880	22,600			10.5	6.5
				2.0	7	9.9	6.0	1,380	23,000	4.2	728	17,300			9.0	7.7
													20,000	18		
Cw	74	91	22	0.5	7	9.9	2.7	387	14,300	2.0	312	15,600			...	...
				1.0	7	9.9	5.1	775	15,200	3.6	629	17,500			...	...
				1.5	9.5	7.3	6.7	1,023	15,300	5.3	819	15,500			10.5	6.5
				2.0	7.5	9.2	8.7	1,356	15,600	6.8	1,111	16,300			9.0	7.7
													16,000	17.5		
Ds	71	81	25	0.5	8.5	8.2	3.1	406	13,100	2.4	331	13,800			...	...
				1.0	6.5	10.7	6.6	714	10,800	4.9	564	11,500			...	...
				1.5	9.5	7.3	7.6	1,032	13,600	6.3	838	13,300			9.5	7.3
				2.0	8	8.7	10.0	1,410	14,100	7.4	936	12,700			9.0	7.7
													13,000	16		

[a] Determined from free drug blood concentration data.
[b] k was calculated from the $(t^1/_2)$ determined graphically.
[c] Expressed as mg. equivalents of SETD, calculated from dose administered.

of these limitations has been described (8). This method takes into account the fact that a plot of the equation

$$\log (dC/dt) = (-k_e t/2.303) + K \qquad (2)$$

theoretically should give a line with identical slope to a plot of log C versus t.

When the amount of drug excreted during each 3-hour interval by individual subjects was plotted according to Eq. 2, the variability of the data was too great to attempt a straight line plot. The results indicate that for this drug and under the conditions of this study an application of Eq. 2 to urine concentrations of single subjects usually requires an accumulation of data over more than 4 equal time increments. When the free drug excreted for each 3-hour interval was averaged for the four subjects, the data tended to follow the requirements of Eq. 2. This is illustrated in Fig. 3. Though this method of plotting the average excreted free drug for several subjects at a single dosage level is not a mathematically rigorous application of Eq. 2, it is a rather good approximation of it (8). The method permits a utilization of all the 3–15-hour data of Table II. The 0–3-hour data are excluded because they do not represent a period of diffusion equilibrium.

The rates of SETD elimination via the urinary tract, as determined by this method, emphasize the data of the first 15 hours and in this respect are more comparable to the blood level data, also obtained during a relatively short period after SETD administration.

Table IV records the values of $(t_{1/2})_u$ as determined by this method and compares these with $(t_{1/2})_b$. The data are seen to be sufficiently alike to suggest that either blood concentration or urinary excretion measurements may be employed to obtain fair approximations of SETD disappearance rate from the tissues. The rates determined by either method correspond closely.

The harmonic mean half-life for urinary excretion at 0.5–2-Gm. doses, as determined from the straight line plots of Fig. 3, is 8 hours. This contrasts with a $(t_{1/2})_b$ of 7.5 hours from blood level data and a $(t_{1/2})_u$ of 10 hours from plots according to Eq. 5. The close adherence to the first-order rate and the relative constancy between individuals of the half-lives and velocity constants for drug disappearance

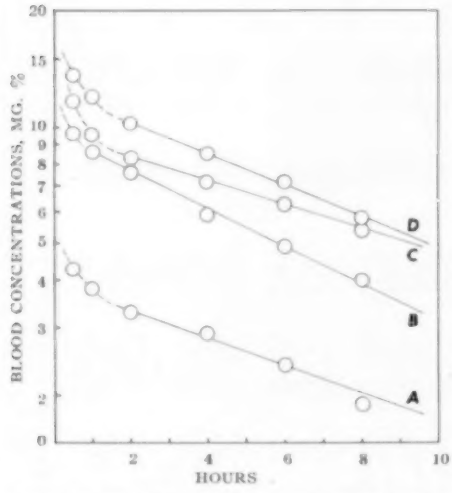

Fig. 1.—Free SETD blood concentrations in subject *Ds* following (A) 0.5, (B) 1, (C) 1.5, and (D) 2 Gm. intravenous injections of the drug.

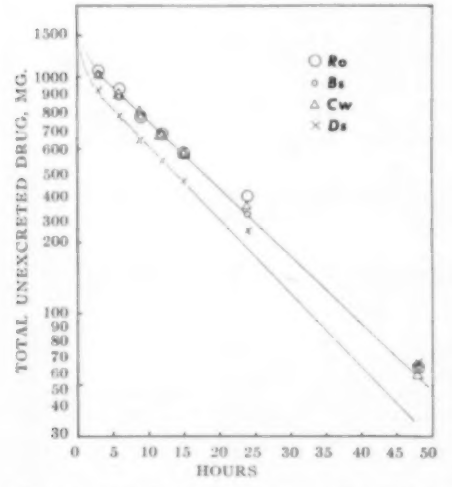

Fig. 2.—A plot of total drug excretion rate for four subjects receiving 1.5 Gm. intravenous SETD, according to Eq. 1.

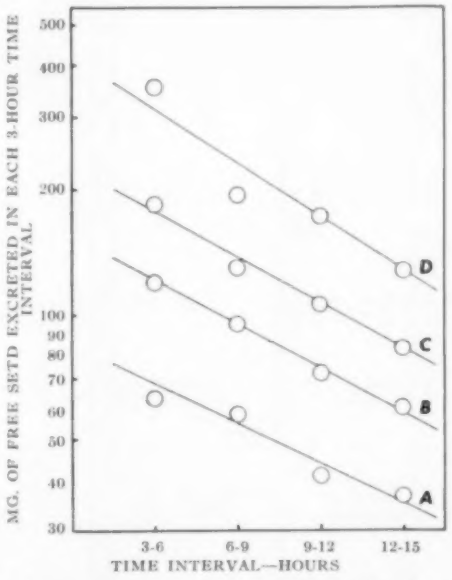

Fig. 3.—Average urinary excretion of SETD for 4 subjects receiving intravenous injections of (A) 0.5, (B) 1.0, (C) 1.5, and (D) 2.0 Gm. plotted according to Eq. 2.

is especially remarkable in view of the variation of urine output in different subjects and at different periods of time. The data suggest that the rate of urinary drug output is not affected appreciably by the amount or rate of urine voided.

Another interesting observation of this study is that the blood concentrations appear to be very sensitive to SETD urinary excretion and distribution processes going on in other tissues. The close correspondence of excretion rates determined by blood and urine studies suggests that the blood concentration-time curves quite accurately describe

TABLE IV.—BIOLOGIC HALF-LIFE FOR SETD IN FOUR SUBJECTS AT FOUR DOSES GIVEN INTRAVENOUSLY, DETERMINED FROM BLOOD CONCENTRATIONS AND URINARY EXCRETION DATA

Dose	$(t^1/_2)_B$[a]	$(t^1/_2)_U$[b]
0.5	8	9.5
1	7	8
1.5	9	8
2	7	7

[a] Harmonic mean computed from data of Table III.
[b] Determined graphically from Fig. 3.

the rate at which SETD disappears from tissues comprising its body space.

Distribution Volume.—If diffusion equilibrium was established in a few minutes following the injections, the intercept value of the ordinate, C_o, of the straight line semilog plot could be used to compute the apparent distribution volume, V_b, from blood concentration data (5, 6) for each dose according to the equation

$$V_b = \text{Dose}/C_o \qquad (3)$$

Since diffusion equilibrium was not attained this rapidly, such a calculation could introduce a considerable error in the determination of V_b. To make such tests more accurately would necessitate determining the amount of drug remaining in the body at various times after diffusion equilibrium was attained. The drug remaining in the body after, for example, 3 and 6 hours, is readily approximated by subtracting from the original doses the amounts appearing in the urine after these times. The free and total drug blood concentrations after 3 and 6 hours are readily determined from the semilog blood concentration *versus* time plots. The fractions of free drug comprising total drug remaining in the body would appear to be related to their respective blood concentrations at any time. The most elegant computations of V_b would involve such correction for the amounts of biotransformed drug. Though this may be done with the data at hand, it is considerably simpler to make the computations with total drug values of blood concentrations, and body drug content at 3 and 6 hours. We have observed that the computations for V_b give essentially the same absolute values irrespective of whether these calculations are for free or total SETD. The simpler computations utilizing total drug blood concentrations and total drug remaining in the body therefore are reported in this paper.

The value of V_b in cc. may then be determined if

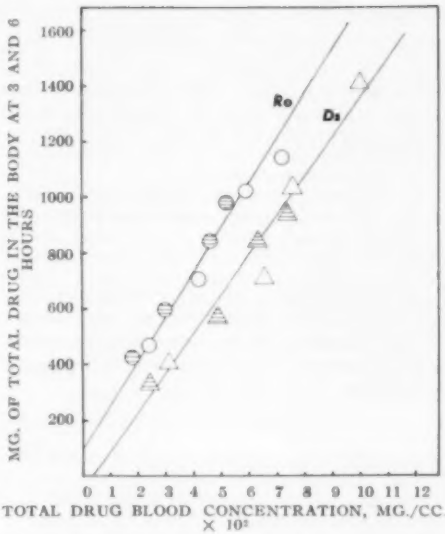

Fig. 4.—A plot of total drug in the body *versus* total drug blood concentrations for subjects *Ro* and *Ds*. Open symbols represent 3-hour data, shaded are 6-hour data.

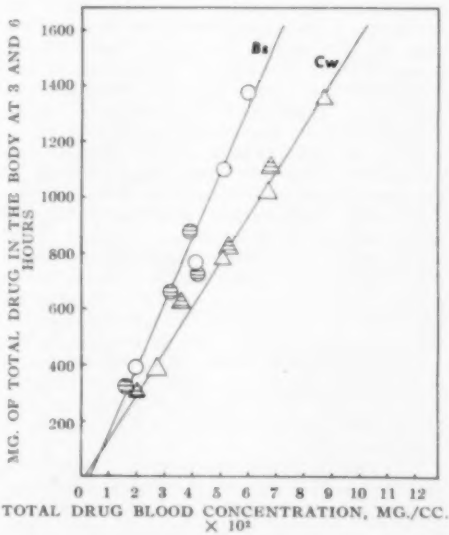

Fig. 5.—A plot of total drug in the body *versus* total drug blood concentrations for subjects *Bs* and *Cw*. Open symbols represent 3-hour data, shaded are 6-hour data.

one assumes

$$V_b = W/C \qquad (4)$$

where, W = mg. of total drug in the body at time t (and theoretically exclusive of any drug in the gastrointestinal tract and bladder), and C = mg./cc. of total drug in the blood at time t, after establishment of diffusion equilibrium.

The values of V_b can be determined analytically at both the 3- and 6-hour blood concentrations, at each dose, for each subject. These values are recorded in Table III. They range from 10,800 to 23,700 cc. with the average V_b for each subject ranging from 13,000 to 20,000 cc. Variations in the values of V_b can be expected when the body volumes of the individuals vary. Correction for subject volumes is made by expressing distribution in volume per cent ($V_b\%$) (6), i. e.,

$$V_b\% = V_b/V_s \qquad (5)$$

where $V_b\%$ is an apparent per cent distribution volume determined from blood concentration data, V_b is expressed in cc. as defined in Eq. 4, and V_s is the subject volume in cc.

For practical purposes one may assume, without introducing appreciable error, that the subject weight in Gm. is effectively equal to the value of V_s, and may be substituted for it. The values of $V_b\%$, calculated to the closest 0.5% are given in Table III. They vary from 16 to 21% with an average of about 18%. Though one might expect $V_b\%$ to vary somewhat in subjects of different age, physique, health, etc., the values show a close correspondence in these four individuals. The values of V_b and $V_b\%$ are only apparent values and are not to be construed to represent the limiting bodily volumes accessible to this drug. This would be true only if the drug concentration was identical in all tissues through which it became distributed. The likelihood of this happening is quite remote. The value of $V_b\%$ may be considered a standard performance index characteristic of this drug in man when determined from blood concentration data. Dominguez (5) has illustrated the theoretical importance of this constant in determinations of absorption and excretion rates. It may be noted that, theoretically, $V\%$ values could be determined for this and possibly other drugs from spinal or other tissue drug concentrations according to concepts similar to those described by Eqs. 4 and 5. These values would have different magnitudes except in the unusual situation in which drug concentrations in the various tissues were identical. These values could all be considered standard performance indexes, describing for the drug in question its distribution and performance characteristics as a family of interrelated scalar units. These would be useful indexes: however, in man it would be difficult to obtain the necessary data for most tissues other than blood.

The apparent distribution volumes of some of the sulfonamides have been reported (14) as determined from plasma concentration data, and may be designated V_p. It should be noted that V_b and V_p may not be of the same magnitude. Where drug penetration of the red blood cells is of a low order, as it is with SETD, V_p and $V_p\%$ have smaller magnitudes than the corresponding values of V_b and $V_b\%$.

It is apparent from Eq. 4 that a plot of W *versus* C should give a straight line through the origin with slope V_b if the assumptions of the equation are correct. The graphic plots of W *versus* C are illustrated in Figs. 4 and 5 for the 3- and 6-hour data. The fact that the 3- and 6-hour data possess essentially the same slopes indicates the approximate attainment of diffusion equilibrium prior to 3 hours after drug administration. This is in accord with the interpretation of the blood concentration data illustrated in Fig. 1. The fact that these data plot essentially as straight lines, with the lines approximately intersecting the origin, is taken as experimental verification of the assumptions inherent in Eq. 4. The results suggest that V_b is essentially independent of the blood concentration through a range of 0–10 mg. %, corresponding in these subjects to total drug in the body of about 0–1500 mg. As a corollary, in this range, the SETD blood concentrations are approximately proportional to the amount of drug in the body.

The linear relationship between W and C in Eq. 4 is of considerable theoretical interest. It is apparent that when this relationship exists, it may be possible to calculate a family of similar lines relating W and C in individuals of variable V_b. Also, if Eqs. 4 and 5 are combined,

$$V_b\% \cdot V_s = W/C \qquad (6)$$

For an individual weighing, for example, 70 Kg., one can readily calculate or determine graphically the interrelationships of W and C after diffusion equilibrium is established. For such an individual one can calculate that each 1 mg. % of SETD in the blood corresponds to about 126 mg. SETD in the tissues. These postulates and calculations result from the assumption that the values of $V_b\%$ for all human subjects lie around 18%. This assumption can be verified only after data have been acquired on a large number of subjects, taking into account such variables as age, weight, health, and sex.

The ability to conveniently measure drug disappearance rate from the tissues by both blood concentration and urinary data, for a single compound, and to demonstrate rates of close correspondence from both sets of data, makes SETD an interesting drug. This, along with other special attributes of SETD (15), suggests its utility in further studies of absorption, distribution, excretion, clearance, dosage form design, and time-dose studies (3). Results of further studies with this drug will be reported. Those related more closely to subjects of concern to the clinician will be reported elsewhere.

SUMMARY

1. Sulfaethylthiadiazole was administered intravenously in doses of 0.5–2 Gm. without difficulty to four healthy adult male human subjects.

2. A first-order rate of drug disappearance from the blood was illustrated through the 2–8-hour interval, after which no further blood concentrations were determined. The biologic half-life, $(t_{1/2})_b$, of the drug ranged from 5 to 9.5 hours

with a harmonic mean of 7.5 hours. The $(t_{1/2})_b$ appeared to be independent of the dose in the range studied.

3. Blood concentration data indicated that approximately 1 hour was necessary to attain "pseudo steady state" diffusion equilibrium following drug administration. Also, these studies indicated that an average of 4 per cent SETD in the blood was in the conjugated form.

4. Urine collections were made up to 48 hours for all subjects and up to 72 hours for some. Essentially all drug was excreted via the urinary route and nearly 100 per cent could be accounted for in the urine within 72 hours. Urinary excretion data indicated that SETD biotransformation averaged about 8 per cent.

5. Urinary data plots of unexcreted drug *versus* time decreased exponentially over an extended time interval, following attainment of diffusion equilibrium. Plots of the data for each subject indicated that $(t_{1/2})_u$ ranged between 9 and 10.5 hours, with a harmonic mean of 10 hours.

6. Semilog paper plots of average drug excreted by the four subjects during each 3-hour interval from 3 to 15 hours emphasize the urinary data of the early time intervals. By this method of plotting, $(t_{1/2})_u$ ranged from 7 to 10 hours, with a harmonic mean of 8 hours.

7. Urinary output of this drug is not affected appreciably by the amount or rate of urine voided.

8. Combined knowledge for SETD of dose administered, blood concentrations, and total elimination via the urinary route permitted a calculation of the apparent distribution volume and apparent per cent distribution volume in the human body.

9. Cartesian coordinate plots of drug in the body *versus* concentration in the blood were approximately linear approaching intersection with the origin. The 3- and 6-hour data plotted fairly well on the same line, verifying the conclusion from blood concentration studies alone that diffusion equilibrium was essentially attained prior to 3 hours following administration. The linear plots also suggest that the magnitudes of the blood concentrations were directly proportional to the amount of drug in the tissues through a blood concentration range of approximately 0–10 mg. per cent. As a corollary, V_b or V_b per cent are essentially independent of the blood concentration through the same range.

10. The average apparent distribution volumes for each subject from blood concentration data, ranged from 13,000 to 20,000 cc. in subjects weighing 81–114 Kg. Average apparent per cent distribution volumes for each subject ranged from 16 to 21 per cent, with an average of 18 per cent.

11. By a generalization of the results of this study, one may derive the equation

$$V_b\% \cdot V_s = W/C$$

which approximates the interrelationships of apparent per cent distribution volume, body volume (or weight), amount of drug circulating in the tissues, and concentration of drug in the blood up to at least 10 mg. per cent, in a human subject after attainment of diffusion equilibrium of the drug.

12. This combined blood concentration and urinary excretion study indicates that for SETD, blood concentrations appear to be very sensitive to drug distribution processes going on in other tissues. The close correspondence of drug disappearance rates from the body suggest that the blood concentration time curves quite accurately describe the rate at which SETD disappears from tissues comprising its body space.

13. For this drug, the technique of administration via the intravenous and oral routes, coupled with straight line semilog paper plots of the respective blood concentration data, appears to permit a close estimation of the period, and time of cessation, of principal absorption following an oral dose.

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A Study of the Lyophilization of Several Pharmaceutical Gums and Suspending Agents*

By LEON LACHMAN† and LEONARD CHAVKIN

Previous reports indicate that there is a marked increase in the rate of solution of most materials that have been lyophilized. Thus, this investigation was undertaken to determine whether several pharmaceutical gums and suspending agents, which are not readily soluble or dispersible in water, would be more rapidly soluble or dispersible in the lyophilized state. Data are presented showing that the rate of solution or dispersion of sodium carboxymethylcellulose 70 L, 70 M, 120 M, 120 H, Veegum, Veegum H.V., Carbowax 6000, acacia, tragacanth, sodium alginate, bentonite, agar, gelatin, pectin and Clear Flo-H starch are markedly increased after lyophilization. For the majority of the compounds studied, no significant change in viscosity or pH was observed when the lyophilized material was reconstituted.

GUMS AND OTHER SUSPENDING AGENTS are commonly employed by pharmaceutical manufacturers in the preparation of various formulations and by retail pharmacists in extemporaneous prescription compounding. The rate of solution or dispersion of these hydrophilic materials is generally very slow. Frequently long periods of heating, stirring, and other manipulations are necessary to accomplish solution or dispersion. The development of a technique of preparing gums and suspending agents in a readily soluble form would materially decrease the time and effort required to prepare emulsions, lotions, suspensions, and similar liquid preparations.

Since previous reports indicated that the rate of solution of most materials in the lyophilized form is substantially increased, the present investigation was initiated to determine the effect of lyophilization on the rate of solution or dispersion of several pharmaceutical gums and suspending agents. The data obtained from this study indicate that the rate of solution or dispersion of several viscosity grades of sodium carboxymethylcellulose, Veegum,® Carbowax® 6000, acacia, tragacanth, sodium alginate, bentonite, agar, gelatin, pectin and ClearFlo-H starch are markedly increased whereas that of methylcellulose 4000 is significantly decreased by this method of drying.

Lyophilization or freeze-drying is now widely used as a commercial operation, but twenty years ago it was scarcely more than a laboratory curiosity. It consists of the rapid freezing of an aqueous solution of a material and subsequent removal of the water by sublimation of the ice under conditions of high vacuum.

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In the early stages of the development of this technique of drying, it was used primarily for the preservation of serums, bacteria, viruses, and cultures (1–6). With the advent of World War II, the extensive use of lyophilization began on a commercial scale. Blood plasma, penicillin, and streptomycin were processed by this method of drying (7). In recent years, water-soluble vitamins (8–10), hormones, enzymes, and other active medicinal agents have been lyophilized (11). Although it is realized that this method of drying affords many advantages, they all contribute to the stability of the finished preparation.

Past studies reveal that the technique of lyophilization has been used for numerous materials. However, only limited attention has been given to the effect of this drying technique on gums and suspending agents. Therefore, this study was undertaken to determine whether it would be feasible to lyophilize gums and suspending agents in order to obtain a readily soluble or dispersible form of these materials.

EXPERIMENTAL

In order to determine whether there is any change in the rate of solution of the lyophilized form of several commonly used pharmaceutical gums and suspending agents, as compared to the original form of these materials, solutions or dispersions of the materials were lyophilized using a Stokes Model 103 LPM manifold freeze dryer (Fig. 1).

Materials

CMC® 70 L, 70 M, 120 M, 120 H—Hercules Powder Co.
Veegum, Veegum, H. V.—R. T. Vanderbilt Co., Inc.
Carbowax® 6000—Union Carbide and Carbon Co.
Acacia U. S. P., tragacanth U. S. P., bentonite U. S. P., agar U. S. P.
Gelatin U. S. P., sodium alginate N. F.

Clear Flo-H Starch (sodium salt of an ungelatinized low substituted starch acid-ester derivative containing carboxylic and sulfonic acid groups)—National Starch Co.
Pectin N. F.
Karl Fischer reagent
Solid carbon dioxide
Ethyl alcohol

Apparatus

Model 103 LPM Stokes manifold freeze dryer
Portable Stokes McLeod gauge 276-AA
Stokes vacuum pump No. 146
Standard motor (two stages) 1 HP
Stokes No. 108 rubber sleeves
Stokes No. 109 "S" exhaust tubes
Stokes No. 20 glass containers
Stokes No. 29 exhaust tube stopper for container No. 20
Stokes No. 129 stopper for container No. 20
Beckman Model G pH meter
Karl Fischer titrimeter
Stop watch

Procedure

I. Lyophilization of the Solutions.—The lyophilizer was prepared for freeze drying by attaching the rubber sleeves to the manifold ports, the exhaust tubes to the rubber sleeves and the exhaust tube stoppers to the exhaust tubes. The vacuum valves

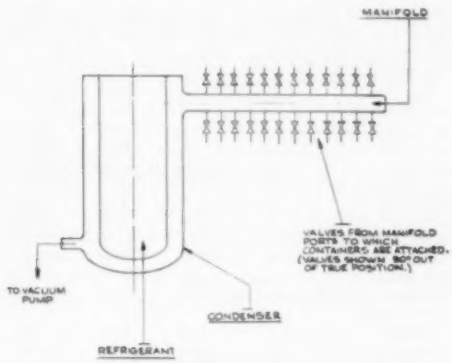
MANIFOLD
VALVES FROM MANIFOLD PORTS TO WHICH CONTAINERS ARE ATTACHED. (VALVES SHOWN 90° OUT OF TRUE POSITION.)
TO VACUUM PUMP
CONDENSER
REFRIGERANT

Fig. 1.—Schematic diagram of manifold type lyophilizer.

to the ports were closed and solid carbon dioxide in ethanol was placed into the condenser. After the condenser had cooled the vacuum was applied.

The solutions of the gums were prepared for drying by placing 25 ml. of sample into the 50-ml. containers and stoppering with rubber closures. The stoppered containers were placed horizontally on two stainless steel trays containing solid carbon dioxide in ethanol. When the samples had frozen as slabs along the vertical axes of the containers, the containers were attached to the manifold. At the time of the attachment of the containers to the manifold, the vacuum in the lyophilizer was essentially 50 microns.

At the start of the drying cycle each container was attached to the manifold by removing the rubber stopper from the container and attaching the container to the manifold by the exhaust tube stopper. Each manifold port vacuum valve was opened as the containers were attached. The low vacuum in the apparatus kept the material in the containers frozen and removed the ice as water vapor which was condensed along the periphery of the condenser. The external heat applied to the containers during the drying process was that of room temperature. The material was allowed to remain on the lyophilizer for a period of twenty-four hours. However, when this period of time was not adequate for complete drying, lyophilization was continued for forty-eight hours. The vacuum was maintained between 40 and 50 microns, and the average temperature of the condenser was −78° during the drying cycle.

At the end of the drying period, the vacuum valve of the condenser was opened and the pump was stopped. This caused dry air to fill the containers, since the moisture in the air entering the lyophilizer condensed along the condenser as it passed through on its way to the containers. The containers were then removed from the exhaust tube stoppers and immediately stoppered under atmospheric conditions with rubber stoppers.

II. Rate of Solution—The solubility test consisted of adding 25 ml. of distilled water at 25° to the container of the lyophilized material and agitating at a constant speed for ten to fifteen seconds. If the material went into solution or formed a dispersion with the appearance of the original solution, the sample was called "immediately soluble," if not, other notations were made accordingly.

III. Viscosity Measurements—The relative viscosity measurements were determined according to the method described by Huyck (12) with slight adaptations. A permanent mark was made approximately 5 cm. below the bulb of a 10-ml. pipet. The solution or dispersion to be tested was drawn into the pipet and the time in seconds for its level to fall from the upper to the lower mark was noted. These measurements were made at 25° relative to distilled water which was used as a reference standard with a viscosity equal to 1.

IV. Moisture Determination.—The moisture content was measured by the Karl Fischer titration method (13). Care was taken to have a closed system during assay so that essentially no atmospheric moisture could interfere with the results obtained.

RESULTS AND DISCUSSION

The rate of solution of the samples immediately after lyophilization and of the lyophilized samples after storage at 40° for three weeks was compared with that of the original material. These data along with the physical appearance of the samples have been tabulated and are presented in Table I.

The data in Table I reveal that for all materials tested, except Methocel® 4000, there was a marked increase in the solubility rate of the lyophilized material as compared with that of the original material. The majority of the lyophilized materials were immediately soluble and a few were soluble within two to fifteen minutes. Furthermore, no special dispersion techniques or equipment were required to accomplish solution. The lyophilized methylcellulose 4000, however, exhibited a substantial decrease in solubility rate. When the lyophilized methyl-

TABLE I.—RATE OF SOLUTION OR DISPERSION OF ORIGINAL MATERIAL AND LYOPHILIZED MATERIAL

Material	Solution, %	Lyophilization Time, Hr.	Solution Rate Before Lyophilization	Solution Rate Immediately After Lyophilization	Solution Rate of Lyophilized Material Stored at 40° for 3 Weeks	Appearance of Solution of Lyophilized Material at 40° for 3 Weeks as Compared to Original Solution
CMC 70 L	1	24	1–2 hr.	Immediate[b]	Immediate	Same
CMC 70 M	1	24	1–2 hr.	Immediate	Immediate	Same
CMC 120 M	1	24	1–2 hr.	Immediate	Immediate	Same
CMC 120 H	1	48	1–2 hr.	Within 5 min.	Within 5 min.	Same
Veegum	4	24	1/2 hr.[d]	Immediate	Immediate	Same
Veegum H. V.	2	24	1/2 hr.[d]	Immediate	Immediate	Same
Carbowax 6000	1	24	1/2 hr.	Immediate	Immediate	Same
Acacia	10	24	1 hr.	Immediate	Immediate	Same
Tragacanth	2	24	24 hr.[c]	Immediate	Within 2 min.	Same
Sodium alginate	2	24	1 hr.	Within 5 min.	Within 15 min.	Same
Bentonite	5	24	24 hr.[d]	Immediate	Immediate	Same
Agar	2	24	1/2–1 hr.	Immediate	Immediate	Same
Gelatin	5	48	1/2 hr.	Immediate	Immediate	Same
Pectin	2	24	1/2 hr.	Within 5 min.	Within 10 min.	Same
Clear Flo-H starch	2	48	1 hr.	Immediate	Immediate	Same
Methocel 4000	1	24	2–4 hr.	Not soluble[a]	Not soluble[a]	Same

[a] Soluble within 48 hours.
[b] Soluble within 10–15 seconds.
[c] Time required for bassorin to swell to its maximum capacity.
[d] Time required for complete hydration.

cellulose 4000 was mixed with water, it formed a ball which floated to the surface. After remaining at room temperature for forty-eight hours, with intermittent agitation, the balled methylcellulose 4000 appeared to form a clear solution.

A few of the materials, when stored at 40° for three weeks, seemed to show a decrease in their solubility rate. This is essentially due to the fact that the moisture content of the lyophilized materials had increased resulting in a change of the physical characteristics of the material. This was borne out by the data in Table IV.

Relative viscosity measurements at 25° were taken on the original solutions, solutions of the material immediately after lyophilization, and on the solutions prepared from lyophilized material stored at 40° for three weeks. As can be seen from Table II, most of the lyophilized materials exhibited a slight decrease in relative viscosity, a few showed no change and one showed rather erratic results as compared to the original solutions. The slight decrease in relative viscosity may be due to the experimental error involved in this method of viscosity measurement. However, the large decrease in relative viscosity of the lyophilized pectin and tragacanth may be due to a change in the physical structure of the lyophilized material as compared to the original material. The erratic results obtained with the lyophilized sodium alginate appear to be due to the fact that when distilled water was added to the lyophilized material, clumping resulted and a few small particles may not have been completely in solution when the viscosity measurements were taken. These particles, if not completely in solution, could have caused a slower flow of the solution through the capillary during the viscosity measurements. This would then result in a higher viscosity reading.

TABLE II.—COMPARISON OF VISCOSITY FOR SOLUTION OF ORIGINAL AND LYOPHILIZED MATERIALS

Relative Viscosity of Water at 25° = 1

Material	Solution, %	Original Solution	Immediately After Lyophilization	Lyophilized Material After 3 Weeks at 40°
CMC 70 L	1	1.8	1.8	1.8
CMC 70 H	1	4.3	4.2	3.9
CMC 120 M	1	6.7	5.9	5.5
CMC 120 H	1	10.1	8.3	8.6
Veegum	4	[a]	[a]	[a]
Veegum H. V.	2	1.6	1.4	1.4
Carbowax 6000	1	1.0	1.0	1.0
Acacia	10	1.4	1.4	1.4
Tragacanth	2	29.9	15.7	12.0
Sodium Alginate	2	21.6	31.4	25.8
Bentonite	5	2.7	2.4	2.6
Agar	2	[b]	[b]	[b]
Gelatin	5	1.4	1.5	1.2
Pectin	2	43.7	18.9	11.2
Clear Flo-H starch	2	7.6	6.4	..
Methocel 4000	1	6.0	5.6	4.1

[a] and [b]—The viscosity could not be measured by the method employed due to the thixotropic properties of the 4% Veegum solutions and the fact that the 2% agar solution was too viscous.

The pH of the original solutions, solutions of the material immediately after lyophilization, and solutions prepared from lyophilized samples stored at 40° for three weeks were measured using a Beckman

TABLE III.—pH OF SOLUTIONS OR DISPERSIONS

Material	Solution, %	Original Solution	Immediately After Lyophilization	Lyophilized Material After 3 Weeks at 40°
CMC 70 L	1	6.70	6.70	6.73
CMC 70 M	1	6.32	6.42	6.58
CMC 120 M	1	6.60	6.84	6.82
CMC 120 H	1	6.57	6.85	6.83
Veegum	4	8.65	8.47	8.79
Veegum H. V.	2	8.37	8.10	8.45
Carbowax 6000	1	6.10	6.30	6.20
Acacia	10	4.21	4.32	4.34
Tragacanth	2	4.27	4.71	4.70
Sodium alginate	2	6.12	6.18	6.08
Bentonite	5	7.52	7.85	7.99
Agar	2	6.49	6.09	5.92
Gelatin	5	6.10	6.00	5.66
Pectin	2	2.90	3.17	2.89
Clear Flo-H starch	2	5.53	5.54	5.58
Methocel 4000	1	6.35	6.69	6.69

Model G pH meter. The results obtained are represented in Table III.

It is evident from Table III that the pH of most solutions of the lyophilized materials are somewhat higher than the original solutions. This is essentially due to the fact that the solutionsu ndergoing lyophilization also undergo a loss of carbon dioxide during desiccation with a subsequent elevated pH of the reconstituted product (14).

The amount of water present in the samples immediately after lyophilization and after storage at room temperature and at 40° was determined by the Karl Fischer titration method (13). As can be noted from the data presented in Table IV, there appears to be a gradual increase in the moisture content of the samples during storage at room temperature and at 40°. This is probably due to the fact that the rubber stoppers, used as closures for the containers holding the lyophilized samples, are not adequate barriers to atmospheric moisture (15).

The critical relationship between stability of the final product and extremely low moisture content was recognized in 1935 (16). It was also reported that as the per cent moisture content of the lyophilized material increased, there was a decrease in the rate and completeness of solubility (17). This is borne out by the results obtained in this investigation and noted in Table I, where it could be seen that for certain samples stored at 40° for three weeks a decrease in the rate of solution was obtained.

CONCLUSIONS

From the results of the lyophilization of the selected natural and synthetic gums and suspending agents studied in this investigation, the following conclusions can be drawn:

1. There is generally a marked increase in the solubility rate of lyophilized material as compared with that of the original material.

2. The solutions of most of the lyophilized materials studied exhibit a slight decrease or no change at all in relative viscosity as compared with that of the original solutions.

3. Most of the solutions of the lyophilized material exhibit a slight rise in pH as compared with that of the original solution. This is probably due to a loss of carbon dioxide during drying.

4. There is a gradual increase in the moisture content of the samples during storage at 40° and at room temperature. This appears to be due to the diffusion of moisture through the rubber stoppers used as closures for the containers holding the lyophilized samples.

The information obtained from this study suggests that the time, effort, and equipment

TABLE IV.—THE EFFECT OF STORAGE ON THE MOISTURE CONTENT OF THE LYOPHILIZED MATERIAL

Material	Moisture Immediately After Lyophilization, %	Time Stored at Room Temperature in Days	Moisture After Storage, %	Increase In Moisture, %	Time Stored at 40° in Days	Moisture After Storage, %	Increase in Moisture, %
CMC 70 L	1.57	50	3.15	1.58	91	5.25	3.68
CMC 70 M	1.64	50	3.42	1.78	91	6.14	4.50
CMC 120 M	1.88	38	4.35	2.47	91	15.20	13.32
CMC 120 H	1.76	39	1.96	0.20	53	3.75	1.99
Veegum	0.86	35	1.35	0.49	96	16.95	16.09
Veegum H.V.	1.30	56	1.34	0.04	96	1.54	0.24
Carbowax 6000	1.10	56	1.14	0.04	28	1.25	0.15
Acacia	1.24	77	1.28	0.04	86	1.39	0.15
Tragacanth	1.32	33	1.94	0.62	86	2.88	1.56
Sodium alginate	1.16	77	2.32	1.16	86	2.39	1.23
Bentonite	0.78	77	1.08	0.30	86	1.25	0.47
Agar	1.55	33	1.63	0.08	90	2.78	1.23
Gelatin	1.10	45	1.27	0.17	60	1.56	0.46
Pectin	1.40	73	4.10	2.70	88	10.01	8.61
Clear Flo-H starch	1.01	39	1.13	0.12	60	1.67	0.66
Methocel 4000	1.16	56	1.19	0.03	96	1.30	0.14

required for the preparation of lotions, emulsions, suspensions, and other related preparations would generally be significantly decreased by the utilization of lyophilized gums and suspending agents. This will be of special value to the retail pharmacist who is concerned with the use of gums and suspending agents in the extemporaneous compounding of prescriptions. Therefore, if various gums and suspending agents were lyophilized in bulk and on a large scale, it appears that the additional cost for the lyophilized material would be overshadowed by the ease and rapidity of preparation of various formulations requiring the incorporation of the above materials.

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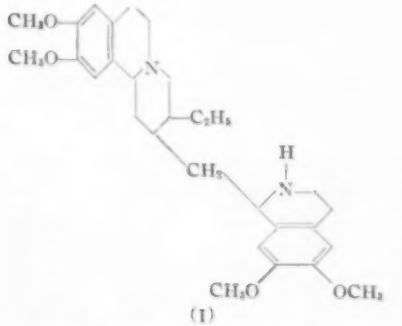
Syntheses of Isoquinoline Derivatives Using 1-(Lithio Methyl) Isoquinoline*

By JOSEPH G. CANNON† and GEORGE L. WEBSTER

As the first step in the development of a general method of synthesis of compounds with possible amebicidal activity, a new organometallic compound, 1-(lithio methyl) isoquinoline, has been prepared. A study of the formation and chemical properties of this compound has been carried out. It has been permitted to react with a variety of functional groups, and derivatives of all resulting compounds have been prepared. Attempts were made to prepare 1-(lithio methyl)-3,4-dihydroisoquinoline, and reasons for the failure to isolate derivatives of this structure are discussed.

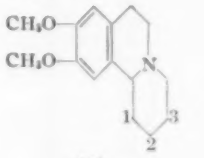
EFFECTIVE chemotherapy in the treatment of extra-intestinal amebiasis was for many years limited almost entirely to the use of emetine (I).



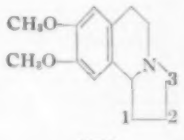
(I)

Currently, it appears that much of the objection to the use of emetine is due not to its lack of efficacy as compared with other agents, but rather to its several unpleasant side effects, its narrow margin of safety, and to its cumulative toxic action on the myocardium (1, 2). Emetine remains a "prototype" compound whose structure could be used as a pattern for the synthesis of more useful and less toxic amebicidal agents.

It has been demonstrated by Pyman (3), Sugasawa (4), and Sugasawa and Kametani (5) that the fused three-ring hexahydrobenzpyridocoline system (II) of emetine is the pharmacophore of the molecule. These workers have prepared several hexahydrobenzpyridocolines, and have found them to be active *in vitro* against the amebal parasite.



(II)



(III)

The investigation reported herein was undertaken as a first step in the development of a

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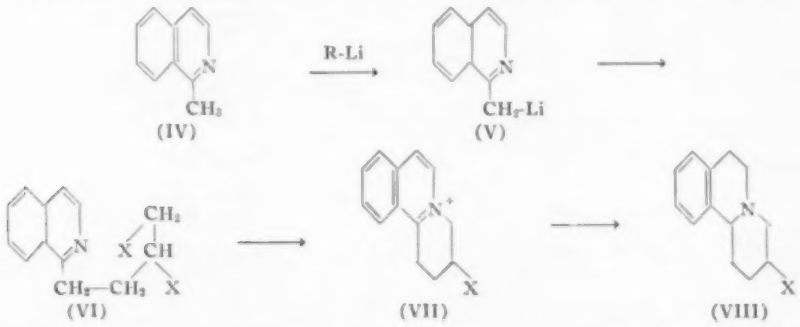
general route for synthesis of benzpyridocolines and for the synthesis of the closely related benzopyrrocolines (III) with functional groups in the 1, 2, or 3 positions, for further elaboration of the molecule into a series of compounds with possible amebicidal activity. The type of synthetic approach envisioned by us beginning with 1-methyl isoquinoline might be illustrated as follows ("X" represents any suitable functional group):



Since studies of the organometallic compounds of 1-methyl isoquinoline have been reported infrequently in the literature, and since we have found no mention of lithium derivatives, the opportunity for an investigation of the formation and chemical properties of 1-(lithio methyl) isoquinoline (V) was apparent. This reagent was prepared, and was permitted to react with a variety of functional groups. Some of the resulting 1-substituted isoquinolines were thought to be amenable to direct ring closure by established methods, and others were convertible to structures that could be cyclized. These isoquinoline compounds can be written as open chain analogs of benzopyrrocoline and benzpyridocoline rings and, it was thought, might possess some pharmacological activity.

Since most amebicidal benzopyrrocolines and benzpyridocolines contain reduced hetero rings, a synthesis beginning with a 3,4-dihydroisoquinoline derivative would be a direct approach. Accordingly, the behavior of 1-methyl-3,4-dihydroisoquinoline in some metalation reactions was investigated and attempts were made to condense it with some organic moieties.

Although studies (6) have demonstrated the pharmacologic value of alkoxyl groups in the benzene ring of some tricyclic systems, the present study was conducted on unsubstituted benzene rings. This was a matter of convenience, but we are of the opinion that the results of our investigation of the lithium derivatives of 1-methyl isoquinoline can be applied to its alkoxylated derivatives.

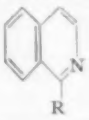
EXPERIMENTAL

1-(Lithio Methyl) Isoquinoline.—Three and one half grams (0.025 mole) of freshly distilled 1-methyl isoquinoline[1] dissolved in 200 ml. of anhydrous ether was placed in a 500-ml., round-bottom three-necked flask, equipped with an inlet tube for nitrogen, a dropping funnel, and an efficient reflux condenser fitted with a calcium chloride-soda lime tube. The flask was seated on a magnetic stirring apparatus, with a "teflon" covered magnetic stirring bar in the flask. After the entire apparatus had been flushed thoroughly with dry nitrogen with a slow stream of nitrogen passing through it, a volume of freshly assayed ethereal solution of alkyl or aryl lithium (7, 8) equivalent to 0.025 mole of the lithium compound was added in a slow stream to the well-stirred contents of the flask. A moderate to vigorous boiling ensued, and the colorless solution turned brick-red. After addition of all the alkyl or aryl lithium solution, the mixture was stirred at room temperature for an additional one and one-half to two hours. At the end of this period, the ethereal solution was a very deep brick-red color, and was opaque; a white granular precipitate was sometimes in evidence. The condensation of 1-(lithio methyl) isoquinoline with the several organic functional groups was immediately carried out in the same apparatus.

Method A.—To the ethereal solution containing 0.025 mole of 1-(lithio methyl) isoquinoline was added, in a slow stream and with stirring, a solution of 0.025 mole of organic halide in about 100 ml. of anhydrous ether. Stirring was continued overnight at room temperature. An additional 0.0125 mole of the halogen compound in about 50 ml. of anhydrous ether was added and the mixture was refluxed vigorously on a steam bath for fifteen minutes or until the color was completely discharged. The apparatus was then removed from the steam bath and, with stirring, 50 ml. of water slowly added. The resulting two clear liquid phases were transferred to a separatory funnel, the aqueous layer was drawn off and discarded, and the ethereal layer was washed three times with water. After drying with anhydrous magnesium sulfate and filtering, the ether was removed on a steam bath, and the brown-black oily residue was distilled under reduced pressure, see Table I.

[1] The authors are indebted to Dr. Howard W. Bond, United States Public Health Service, for the gift of 100 Gm. of 1-methyl isoquinoline

TABLE I.—SUBSTITUTED ISOQUINOLINES



Compound	Method of Prepn.	Reactant	Yield %	B. p. or M. p.[d]	Analysis[a]	Density	N_D^{20}
1-Isobutyl isoquinoline	"A"	Isopropyl iodide	39.6	95–100/1 mm.	For $C_{13}H_{15}N$: N, calcd., 7.56; Found, 7.60, 7.68	1.025 (28.5°)	1.5799
.... Picrate[b]	...	...	...	168–171	For $C_{19}H_{17}N_4O_7$: N, Calcd., 13.52; Found, 13.34	...	...
.... Methiodide[c]	...	...	...	195–197	For $C_{14}H_{18}NI$: I, Calcd., 38.78; Found, 39.01, 38.90	...	...
1-(3-Butenyl) isoquinoline	"A"	Allyl bromide	66	82– 90/1 mm.	For $C_{13}H_{13}N$: N, Calcd., 7.64; Found, 7.43, 7.75	1.043 (26°)	1.5927
R = $-CH_2-CH_2-CH=CH_2$ .... Picrate	...	...	...	151–4	For $C_{19}H_{15}N_4O_7$: N, Calcd., 13.58; Found, 13.30	...	...
... Methiodide	...	...	...	134–6	For $C_{14}H_{16}NI$: I, Calcd., 39.03; Found, 39.12, 38.83	...	...
1-(3-Methyl-3-butenyl) isoquinoline	"A"	Methallyl chloride	63.5	100–10/1 mm.	For $C_{14}H_{15}N$, N, Calcd. 7.11; Found, 7.14, 7.25	1.003 (27°)	1.5867
R = $-CH_2-CH_2-C(CH_3)=CH_2$ .... Picrate	...	...	...	130–131	For $C_{20}H_{17}N_4O_7$: N, Calcd., 13.15; Found, 13.10	...	...
... Methiodide	...	...	...	163–165	For $C_{15}H_{18}NI$: N, Calcd., 4.13; Found, 4.19, 4.26	...	...
Diphenyl, isoquinaldyl carbinol	"B"	Benzophenone	100	136–138	For $C_{23}H_{19}NO$: N, Calcd., 4.30; Found, 4.35, 4.34	... ...	... ...
R = $-CH_2-C(OH)(C_6H_5)_2$							

(*Continued on Page 419*)

[a] Nitrogen of picrates and carbon, hydrogen by Drs. Weiler and Strauss, Oxford, England. Other nitrogen by Kjeldahl method. Halogen by a Volhard method.
[b] Picrates recrystallized from 95% ethanol.
[c] Methiodides recrystallized from absolute ethanol or absolute ethanol-anhydrous ether.
[d] All melting points uncorrected.
[e] Hydrochlorides prepared by treatment of the heterocyclic base with anhydrous ethereal hydrogen chloride. A large excess of hydrogen chloride was avoided as this promoted the separation of the hydrochloride as a difficultly crystallizable gum.

.... Picrate	...	...	...	183–185	For $C_{29}H_{21}N_4O_8$: N, Calcd., 10.10; Found, 9.8	...	...
.... Hydrochloride[e]	...	...	...	202–204	For $C_{23}H_{20}NOCl$: Cl, Calcd., 9.83; Found, 9.75, 9.77	...	...
Methyl, isoquinaldyl ketone	"C"	Ethyl acetate	35	B. p 120–130/1 mm.; m. p. 79–81	For $C_{12}H_{11}NO$: N, Calcd., 7.56; Found, 7.41, 7.36	...	...
R = $-CH_2-\overset{O}{\overset{\|}{C}}-CH_3$							
.... Picrate	...	...	...	199–202	For $C_{18}H_{14}N_4O_8$: N, Calcd., 13.53; Found, 13.70	...	...
.... Copper chelate	...	...	...	197–199	For $C_{14}H_{20}N_2O_2Cu$: Cu, Calcd., 14.71; Found 13.93	...	...
Isopropyl, isoquinaldyl ketone	"C"	Ethyl isobutyrate	32.5	130–134/1 mm.	For $C_{14}H_{15}NO$: N, Calcd., 6.57; Found, 7.01, 6.69	1.126 (26°)	1.6545
R = $-CH_2-\overset{O}{\overset{\|}{C}}-\overset{CH_3}{\overset{\diagup}{\underset{\diagdown}{CH}}}\underset{CH_3}{}$							
.... Picrate	...	...	...	183 decompn.	For $C_{20}H_{17}N_4O_8$; N, Calcd., 12.60; Found, 12.30	...	...
.... Hydrochloride	...	...	...	144–146 decompn.	For $C_{14}H_{16}NOCl$: Cl, Calcd., 14.2; Found, 14.6, 14.4	... ...	... ...
.... Copper chelate	...	...	...	189–192	For $C_{28}H_{32}N_2O_2Cu$: Cu, Calcd., 13.02; Found, 13.28	...	...
Beta-(1-isoquinolyl) ethylmalonic ester	"D"	Ethyl (bromo methyl) malonate	16.1	120–130/1 mm.	...		1.4939
R = $-CH_2-CH_2CH\underset{\diagdown}{\overset{\diagup}{}}\underset{COOC_2H_5}{}$							
.... Picrate	...	...	...	190–193	...	...	...
Beta-phenyl gamma-(1-isoquinolyl) butyrophenone hydrochloride	"C"	Benzal-acetophenone	58.6	144–146	For $C_{25}H_{22}NOCl$: Cl, Calcd., 9.00; Found, 8.89, 9.28. N, Calcd., 3.61; Found, 3.66, 3.72	...	...

(*Continued on Page 420*)

TABLE I.—(*Continued*)

Compound	Method of Prepn.	Reagent	Yield %	B. p. or M. p.[d]	Analysis[e]	Density	N_D^{25}
R = $-CH_2-CH(C_6H_5)-CH_2-C(=O)-C_6H_5$							
Copper chelate Isoquinaldyl 3-hydroxypropyl ketone	"C"	Butyro-lactone	25.8	86–88	For $C_{28}H_{28}N_2O_4Cu$: Cu, Calcd., 12.21; Found, 11.51. N, Calcd., 5.38; Found, 4.92, 5.08	...	...
R = $-CH_2-C(=O)-CH_2-CH_2-CH_2(OH)$							
1, 1-Diphenyl-2-(1-isoquinolyl)-ethylene	...	...	66	127–128	For $C_{23}H_{17}N$: N, Calcd., 4.55; Found, 4.33, 4.42 C, Calcd., 89.9; Found, 89.6. H, Calcd., 5.54; Found, 5.62	...	...
R = $-CH=C(C_6H_5)_2$							
.... Picrate	...	...	...	189–191	For $C_{29}H_{19}N_4O_7$: N, Calcd., 10.43; Found, 10.10	...	...
Beta-(1-isoquinolyl) ethylmalonic ester	"D"	Ethyl methyl-enema-lonate	14.1	132–140/1.5 mm.	...	...	1.4950
.... Picrate	...	...	...	190–193	...	...	...

The condensation products of unsaturated halogen compounds retained their unsaturated character, as evidenced by positive tests for double bonds with potassium permanganate and with bromine in carbon tetrachloride. These tests were not given by 1-methyl isoquinoline.

Method B.—To a well-stirred solution of 0.025 mole of 1-(lithio methyl) isoquinoline in about 200 ml. of anhydrous ether was added, in a slow stream, 4.55 Gm. (0.025 mole) of redistilled benzophenone dissolved in about 100 ml. of anhydrous ether. Stirring at room temperature was continued for about two hours after addition of the benzophenone, then 50 ml. of water was added slowly and with stirring. The tan ethereal layer was separated and was washed three times with small portions of water. On standing at room temperature, large white needles began to separate from the solution. About half of the ether was permitted to evaporate; the remaining solution was thoroughly cooled in an ice-water mixture, and the crystals were collected on a suction filter. They were recrystallized from anhydrous ether, producing small, white, felted needles, see Table I.

Method C.—To 0.025 mole of 1-(lithio methyl) isoquinoline in about 200 ml. of anhydrous ether was added, in a slow stream and with stirring, 0.025 mole of freshly distilled ester dissolved in about 100 ml. of anhydrous ether. The mixture was stirred at room temperature for an additional two and one-half hours. The reaction flask was cooled by immersion in an ice-water bath, and 50 ml. of 6 *M* hydrochloric acid was added cautiously and with efficient stirring. The ethereal layer was separated and was extracted twice with 25-ml. portions of 6 *M* hydrochloric acid, the extracts being added to the original aqueous layer. The combined aqueous extracts were treated with solid sodium bicarbonate until effervescence ceased; the resulting brown oil was taken up in ether. The water layer was extracted twice more with 50-ml. portions of ether, which were added to the main ether solution. The combined ether extract was washed once with 25 ml. of distilled water, dried over anhydrous magnesium sulfate and filtered. The ether was removed on a steam bath and the residue was distilled under reduced pressure. Methyl isoquinaldyl ketone crystallized after distillation, and was recrystallized from isopropyl ether. Attempts to crystallize isopropyl, isoquinaldyl ketone were not successful; this compound appeared to decompose slightly on distilling. Isoquinaldyl, 3-hydroxypropyl ketone could neither be distilled nor crystallized. It did not form a stable picrate but it was isolated as the copper (II) chelate. All of the ketones had vivid yellow to greenish yellow colors, and gave deep blue-green colors with alcoholic ferric chloride solution, see Table I.

Method D.—This method was identical with Method "A" except that no additional reagent was added during the course of the reaction, the molecular equivalents of reagent and 1-(lithio methyl)-isoquinoline being maintained at a one to one ratio, see Table I.

1,1-Diphenyl-2-(1′-isoquinolyl) Ethylene.—Two grams of recrystallized diphenylisoquinaldyl carbinol was dissolved in 23 ml. of glacial acetic acid and 3.5 ml. of concentrated sulfuric acid was added. The bright yellow solution was boiled under reflux for five minutes, then was cooled to room temperature, and about 125 ml. of water was added. Solid sodium carbonate was added until effervescence ceased; a yellow gum was thrown from solution which was washed several times with distilled water and induced to crystallize after some time. This solid was recrystallized from aqueous dioxane. A mixed melting point determination with diphenyl-isoquinaldyl carbinol showed a depression, see Table I.

Copper (II) Chelates of the Alkylisoquinaldyl Ketones.—A portion of the ketone was added to an excess of a saturated aqueous solution of cupric acetate contained in a test tube and intimately mixed for fifteen or twenty minutes. A dark, granular solid was formed which was collected on a suction filter and washed with copious amounts of distilled water after which it was air dried and recrystallized from 95% ethanol. All of the chelates were analyzed for copper by a micro method (9) consisting of combustion of a weighed sample of the compound in the presence of concentrated nitric acid, and weighing the residual cupric oxide, see Table I.

Hydrochloride of beta-Phenyl-gamma-(1′-isoquinolyl) Butyrophenone.—1-(Lithio methyl)-isoquinoline was permitted to react with benzalacetophenone according to Method "C." The ethereal reaction mixture was washed twice with water and treated with 100 ml. of a mixture of one volume of concentrated hydrochloric acid and two volumes of water. The hydrochloride precipitated as a light tan solid. It was collected on a suction filter, washed with several portions of ether and recrystallized twice from a mixture of absolute ethanol and anhydrous ether. The white final product gave a positive Beilstein test for halogen and, in alcoholic solution, formed a copious precipitate with alcoholic silver nitrate. It produced an orange precipitate with 2, 4-dinitrophenylhydrazine test solution, see Table I.

Attempted Preparation of 1-(Lithio methyl)-3, 4-Dihydroisoquinoline.—Seven and one-fourth grams (0.05 mole) of freshly distilled 1-methyl-3, 4-dihydroisoquinoline was dissolved in 125 ml. of anhydrous ether in the reaction apparatus previously described. An ether solution containing 0.05 mole of phenyl lithium or *n*-butyl lithium was added slowly. After two hours' stirring at room temperature the mixture was a deep, red-brown color; stirring was continued for from three to eight hours more. In separate experiments, methyl iodide, isopropyl iodide, and benzophenone (0.05 mole dissolved in ether) were added to the mixture. In each instance, the red-brown color was discharged on addition of the reagent. Stirring was continued for periods of time varying from two to eighteen hours and the reaction mixtures were refluxed up to eight hours on a steam bath. The ether solvent was replaced by 1, 4-dioxane in some experiments and the dioxane solutions were refluxed on a steam bath.

At the conclusion of the reflux periods, 50 ml. of water was added to the flask with stirring and the white precipitate which usually was present dissolved. The organic layer was separated, washed twice with distilled water, dried over anhydrous magnesium sulfate and filtered. The solvent was removed on a steam bath and the dark colored liquid residue was distilled under reduced pressure. The starting material, 1-methyl-3, 4-dihydroisoquinoline (identified by its boiling point, refractive index, and the melting point of its picrate) was recovered in yields which were often almost quantitative. In no instance could an identifiable condensation product be isolated.

DISCUSSION

In those experiments where, for purposes of comparison, both phenyl lithium and *n*-butyl lithium were used to form 1-(lithio methyl) isoquinoline little difference in the yield of the final product or in the time and course of the reaction was noted. *n*-Butyl lithium was chosen as the metalating agent in this work because of the more accurate assay method available for it which determines only organometallic basicity. The phenyl lithium assay is an index of total (organometallic plus inorganic hydroxide) basicity.

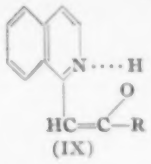
The isolation of a quantitative yield of the condensation product of 1-(lithio methyl)-isoquinoline and benzophenone indicated that the exchange of hydrogen and lithium between 1-methyl isoquinoline and *n*-butyl lithium was quantitative and that the yields in other condensations were dependent on the nature of the substance added to 1-(lithio methyl)-isoquinoline.

An ethereal solution of 1-methyl isoquinoline was stirred at room temperature for two hours with an equimolecular amount of *n*-butyl lithium; the mixture was hydrolyzed with water; the ether layer was separated and dried; and the ether was removed and the residue was distilled. A quantitative yield of 1-methyl isoquinoline was recovered, indicating that there had been no addition of R-Li to the heterocyclic ring under the conditions used as has been reported for some methylated nitrogen heterocycles under somewhat analogous experimental conditions (10, 11).

Apparently addition occurred only on the methyl group of 1-methyl isoquinoline. A Bischler-Napieralsky cyclization of isovaleryl beta phenyl ethyl amine followed by aromatization of the resulting 3, 4-dihydroisoquinoline led to the same product as did the condensation of 1-(lithio methyl)-isoquinoline with isopropyl iodide, namely 1-isobutyl isoquinoline.

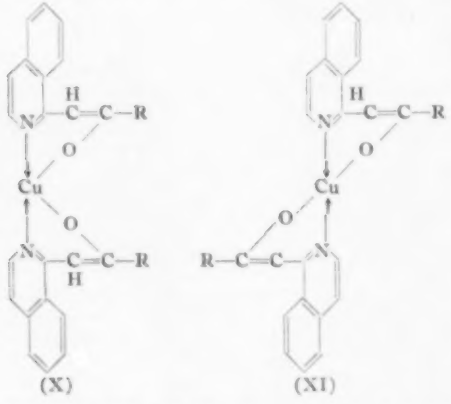
The deep colors produced with ferric chloride and the failure to form carbonyl derivatives indicated that the isoquinaldylalkyl ketones existed partly if not chiefly in the enolic form. Predominance of the enol over the ketonic tautomer is explainable on a twofold basis: the enolic double bond is conjugated with the aromatic ring system, which should increase the stability of the enol; and the enol should be capable of chelating with the ring nitrogen

through a hydrogen bond, formula IX.



(IX)

The preparation of copper (II) complexes of the isoquinaldylalkyl ketones furnished convenient derivatives of the compounds and substantiated our belief that the ketones possessed enolic character. The complexes probably have a chelate structure (X) (the *cis* form, analogous to structures proposed by Goldberg (12) for copper complexes in the quinoline series), or (XI) (the *trans* form).

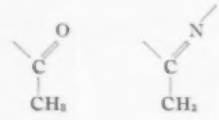


(X) (XI)

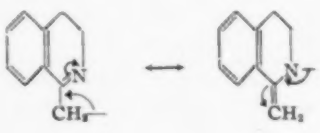
Although Goldberg (without apparent experimental evidence) represented copper (II) chelates of enols of quinaldylalkyl ketones as being *cis*, Mellor (13) stated that the *cis* configuration of internal copper complexes containing unsymmetrical chelate groups is an unstable one, the *trans* form predominating. The copper chelates reported in the present paper varied in color from golden brown to dark purple. In ethanolic solution they produced no deep blue color with ammonia, as did the non-chelate copper complexes of isoquinoline and 1-methyl isoquinoline.

A reason for the apparent failure to prepare 1-(lithio methyl)-3,4-dihydroisoquinoline is suggested by a comparison of the structures of 1-methylisoquinoline and of 1-methyl-3,4-dihydroisoquinoline. The methyl hydrogens of 1-methylisoquinoline are quite liable to attack by a nucleophilic reagent since the anion produced by the loss of a hydrogen ion from the methyl group is capable of resonance into both the hetero ring and the benzene ring, and much stability is achieved.

The methyl group of the dihydro compound is formally similar to an "active methyl" group alpha to a carbonyl function:

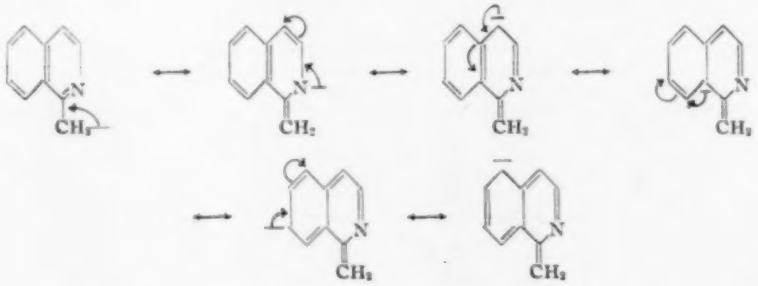


Its carbanion can enter into resonance with the ring nitrogen, but probably not to any significant extent with remainder of the ring system; thus the resonance stabilization of a 1-methyl carbanion of 3, 4-dihydroisoquinoline is much less than for the full aromatic ring:



It is possible that the relatively smaller amount of resonance stabilization of 3, 4-dihydroisoquinoline prevented a metalation of the methyl group. It is, however, difficult to explain the red color obtained when organolithium reagent was added to 1-methyl-3, 4-dihydroisoquinoline. Usually, only those lithium compounds are highly colored in which the metal is attached to a carbon in direct union with an aromatic ring. This might suggest some metalation of the heterocyclic ring at position four, although no derivative of such a structure could be isolated.

Beta-(1-isoquinolyl)-ethylmalonic ester, which was prepared by condensation of 1-(lithio methyl)-isoquinoline with methylene malonic ester and by condensation with (bromo-methyl) malonic ester (14), has been prepared by Boekelheide and Sieg (15) by a Michael addition of sodio malonic ester to 1-vinyl isoquinoline. The refractive index of our product was not identical with that reported by them, but the boiling point of the free base and the melting point of its picrate were identical.



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Cardiac Glycosides of *Euonymus Atropurpurea* Jacq. II.*

A Study of the Structure of Euatroside and Euatromonoside

By C. A. BLISS† and EGIL RAMSTAD

Glycoside *c-1* obtained in a previous investigation from *Euonymus atropurpurea* (1) has been subjected to structural studies. By enzymatic hydrolysis, the glycoside, which was subsequently designated as euatroside, was shown to be a β-glucoside of euatromonoside, a secondary glycoside. The sugar portion of euatromonoside is arabinose. Chromatographic evidence revealed that euatroside contained only two sugars, namely one molecule of arabinose, and one of glucose. Chromatograms of the genins of euatroside and euatromonoside furnished evidence that the aglycon portion is digitoxigenin. From the data obtained the structures of digitoxigenin arabinosido-β-glucoside and digitoxigenin arabinoside are proposed for euatroside and euatromonoside, respectively.

THE SEPARATION of a series of cardiac glycosides from *Euonymus atropurpurea* and the isolation of 12 mg. of one of these glycosides (*c-1*) has been described in a previous communication (1). In a continuation of this work a study of the structure of glycoside *c-1* has been carried out. The glycoside was split by acidic and enzymatic hydrolysis and the hydrolysate investigated by paper chromatography. Information as to the nature of the glycosidic linkages was obtained by means of enzymatic hydrolysis. A secondary glycoside was formed by the enzymatic hydrolysis and could be studied further by acid hydrolysis. The genuine glycoside (*c-1*) and the monoside derived from it were named *euatroside* and *euatromonoside*, respectively.

EXPERIMENTAL

Materials, Reagents, and Procedures

Reference Materials.—Chemically pure arabinose, ribose, xylose, glucose, rhamnose, 4-methyl glucose, digitoxin U. S. P. reference standard, and digoxin U. S. P. reference standard.

Enzymes.—Emulsin was prepared from sweet almond by ammonium sulfate precipitation methods. Invertase was obtained from a commercial source (Bios Laboratories). Strophanthobiase was prepared from *Strophanthus Kombe* seeds[1] by the method of Stoll and Renz (2).

Paper.—Whatman No. 1.

Solvent Systems.—For chromatography of euatroside, Solvent System 1: chloroform 87 parts, ethanol 7 parts, benzene 3 parts, and formamide 3 parts. Excess formamide was removed.

For chromatography of aglycons and secondary glycosides (3), Solvent System 2: chloroform 40 parts, benzene 60 parts, and formamide sufficient for saturation. Excess formamide was removed.

For chromatography of sugars, Solvent System 3: ethyl acetate 8 parts, pyridine 2 parts, and water 1 part.

For chromatography of sugars (4), Solvent System 4: water saturated with boric acid 30 parts and acetone 10 parts.

For chromatography of sugars, Solvent System 5: *n*-Butanol 4 parts, acetic acid 1 part, water 1 part.

Chromatographic Procedures.—All paper-chromatographic runs were carried out by descending technique. In the runs in which Solvent System 1 was used, the filter paper was impregnated by passing the paper quickly through a 10% solution of forma-

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[1] Courtesy of S. B. Penick and Co., New York.

mide in acetone, followed by blotting the sheet between filter paper and allowing the acetone to evaporate. In the experiments in which Solvent System 2 was used the filter papers were impregnated in the same manner with the exception that 30% formamide in acetone was used. The materials to be chromatographed were then applied immediately to the treated papers.

Reagents.—Trichloroacetic Acid-Hypochlorite Reagent (TCAA Reagent) (1): the chromatograms were inspected under ultraviolet light. This reagent was used to locate the digitaloids on the chromatograms.

The *p*-Anisidine Hydrochloride Reagent (5): the *p*-anisidine HCl reagent was prepared by dissolving 4 Gm. of *p*-anisidine HCl in 10 ml. of water. Ten ml. of ethyl alcohol was added, and the volume was adjusted to 120 ml. with *n*-butanol.

The location of the sugars on the chromatogram was established by spraying the chromatogram with the *p*-anisidine HCl reagent and heating it at 85–90° for two to three minutes. Overheating or prolonged exposure of the strip to heat causes the red pentose spots to turn dark brown and produces an overall darkening of the sprayed paper so that the differentiation of the pentose spots from the hexose spots becomes difficult.

Enzymatic Hydrolysis of Euatroside

With Emulsin, Strophantobiase, and Invertin.—Enzymatic hydrolysis of euatroside was carried out on three chromatographic strips spotted with several micrograms of euatroside. Five lambdas of one of three enzyme solutions, *viz.* 1% emulsin, 5% strophanthobiase, and 5% invertase, were each superimposed on different spots of the glycoside.

The strips were placed in a horizontal position at 20–25° in a water-saturated atmosphere to prevent drying of the enzyme solution on the paper. After twenty-four hours the strips were dried and chromatographed with Solvent System 3. A strip spotted with glucose was run simultaneously in the same chamber. The strips treated with emulsin or with strophanthobiase had produced a sugar with an identical R_f value of that of glucose, while the strip treated with invertase displayed no spot of free sugar.

Strophanthobiase.—Five hundred micrograms of euatroside suspended in 1 ml. of water were hydrolyzed in a small test tube with 1 ml. of a 5% strophanthobiase solution. After a period of nineteen days the hydrolyzing mixture was separated into three fractions as follows: the aqueous suspension was first shaken several times with 0.5 ml. of chloroform, the chloroform fractions were combined and evaporated to dryness under nitrogen. The aqueous fraction remaining was dried *in vacuo* and the residue then extracted with three 0.05-ml. portions of alcohol. The combined ethanol fractions were in turn evaporated to dryness under nitrogen. The final extraction of the residue was carried out in a similar fashion with three 0.5-ml. volumes of water. The chloroform-soluble fraction, E_m, and the alcohol-soluble fraction, *A*, were each dissolved in 50 lambdas of alcohol while the aqueous fraction, *W*, after drying *in vacuo*, was dissolved in 50 lambdas of water.

Chromatography of these three fractions (Solvent System 3) with a control strip of glucose alongside showed that fraction *W* and fraction *A* produced spots with an R_g value of 1 (ratio of the distance traveled by unknown to distance traveled by glucose). Fraction E_m furnished no sugar spots. The identity of glucose was confirmed by running a mixed chromatogram of glucose and fraction *A*.

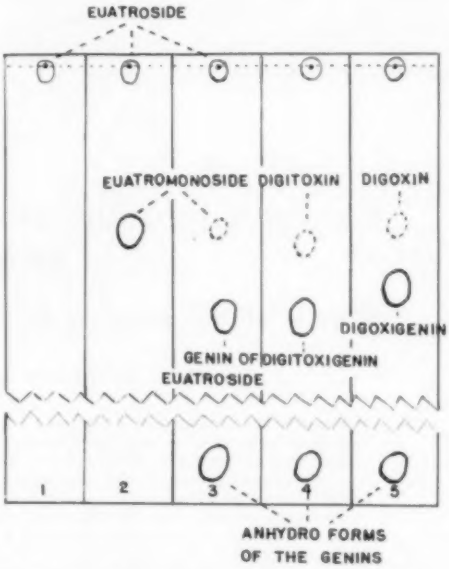


Fig. 1.—Chromatographic separation of Euatroside and the water-insoluble hydrolytic products of Euatroside, of digitoxin (U. S. P. Standard), and of digoxin (U. S. P. Standard). (1) Unhydrolyzed Euatroside; (2) after hydrolysis of Euatroside with strophanthobiase; (3) after Mannich's hydrolysis of Euatroside; (4) after Mannich's hydrolysis of digitoxin; (5) after Mannich's hydrolysis of digoxin.

Another series of four chromatograms was run for twenty-four hours with fractions *W*, *A*, and E_m along with a control chromatogram of unhydrolyzed euatroside. The system consisted of a 10% formamide-impregnated paper and Solvent System 1. Fraction *A* and the control strip showed one spot of euatroside. Fraction E_m contained only a trace of euatroside, while fraction *W* contained none of the β-glucoside. No secondary glycoside or aglycon was visible on the formamide-impregnated papers. However, upon running a duplicate set of chromatograms for a period of two hours only, it became possible to locate a lower glycoside, which was designated as euatromonoside, on the strips containing fraction E_m and fraction *A*. The control strip with euatroside and with fraction *W* showed no euatromonoside spot. Under these conditions, euatromonoside possessed an R_f value of 0.75; euatroside had migrated only a few centimeters from the point of origin.

Mannich Hydrolysis of Euatroside.—A mild but relatively complete hydrolysis, first described by Mannich and Siewert (6), was carried out with the following materials: (*a*) euatroside, 500 micrograms,

(*b*) fraction E_m, which contained euatromonoside, (*c*) digitoxin U. S. P. reference standard, 3 mg., and (*d*) digoxin U. S. P. reference standard, 3 mg. One-half milliliter of 1% HCl in acetone was used as the hydrolyzing medium. Length of hydrolysis was twenty-one days after which 0.5 ml. of water was added to each solution and the acetone was evaporated. Each of the four aqueous mixtures was then shaken out with chloroform in order to separate the aglycons from the sugars present in the hydrolysates. The aqueous fractions were concentrated and set aside. The chloroform fractions were evaporated to dryness and 50 lambdas of alcohol were added to the residues consisting of the aglycons of euatroside and of euatromonoside, while 0.3 ml. of alcohol was added to the aglycons of digitoxin and digoxin.

By the use of Solvent System 2 and filter paper impregnated with 30% formamide in acetone (3) the genins and secondary glycosides could be separated. Five strips, each spotted with one of the following materials, were run: (*a*) unhydrolyzed euatroside, (*b*) fraction E_m, containing euatromonoside, and (*c*, *d*, and *e*) the chloroform-soluble fractions from the Mannich hydrolysis of euatroside, digitoxin, and digoxin, respectively.

Irrigation of the strips was continued for a period of three hours, or until the solvent front reached within a few centimeters from the bottom of the strip. All spots listed in the schematic representation of the results in Fig. 1 displayed a bright yellow fluorescence in ultraviolet light with the exception of the spots of digoxin and its genin, which fluoresced intensely blue and, of course, had different R_f values.

When the aglycon fractions produced by the Mannich hydrolysis of fraction E_m and from euatroside were chromatographed under the same conditions as above, they gave chromatograms identical to Strip 3, Fig. 1.

Chromatographic Study of Sugar Fractions Obtained by Hydrolysis of Euatroside and of Euatromonoside

Monoses.—Solvent System 3 was used for paper-chromatographic analysis of the sugars in the following materials: (*a*) unhydrolyzed euatroside, (*b*) water-soluble fraction from the Mannich hydrolysis of euatromonoside, (*c*) water-soluble fraction obtained by the Mannich hydrolysis of euatroside, (*d*), rhamnose, (*f*) glucose, (g) digitoxose, (*h*) ribose, (*i*) arabinose, (*j*) xylose, (*k*) 4-methylglucose. Equilibration was twelve hours and irrigation was continued for four hours and for twenty-four hours. Unhydrolyzed euatroside gave no spot of free sugar. Table I shows the R_g value of each spot on each strip and the color produced with the *p*-anisidine HCl reagent.

A mixed chromatogram of arabinose and the sugar fraction obtained from the hydrolyzed euatroside produced only one pentose spot by use of Solvent Systems 3, 4, and 5. The two latter solvent systems gave R_g values of 1.1 and 1.4, respectively, for arabinose.

Disaccharide.—The low R_g value of spot No. 1 from the Mannich hydrolysis of euatroside (Table I) was indicative of a saccharide. The sugar fraction was chromatographed alongside sucrose (disaccharide) and raffinose (trisaccharide) for forty-eight hours. Under these conditions raffinose showed a low R_g value (0.04) while the R_g values of sucrose and the unknown saccharide were much higher (0.35 and 0.27, respectively).

TABLE I.—THE R_g VALUES AND COLORS OF REFERENCE SUGARS AND SUGARS PRODUCED BY THE MANNICH HYDROLYSIS OF EUATROSIDE AND EUATROMONOSIDE (SOLVENT SYSTEM 3)

Material Spotted	Spot No.	Color[a] White Light	Color[a] UV Light	R_g[b] Value
Ribose	1	Red	Red	4.1
Arabinose	1	Red	Red	1.8
Xylose	1	Red	Red	2.5
Glucose	1	Brown	Brown	1.0
4-Methyl-glucose	1	Red brown	Brown	2.9
Mannich Hydrolyzed Euatroside	1	Brown	Yellow	0.2
	2	Brown	Brown	1.0
	3	Red	Red	1.8
Mannich Hydrolyzed Euatromonoside	1	Red	Red	1.8
Digitoxose	1	Brown	..	8.0
Rhamnose	1	Brown	Brown	3.4

[a] Colors produced by reaction of sugars with the *p*-anisidine HCl spray reagent.
[b] R_g = distance traveled by sugars/distance traveled by glucose.

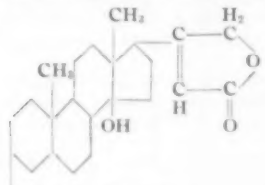
Ratio of Glucose to Arabinose in Euatroside.—Standard solutions consisting of glucose and arabinose in the ratios of 3:1, 2:1, and 1:1 were prepared. These solutions were then spotted, each on separate chromatographic strips, and chromatographed along with a strip containing the sugar fraction from the Mannich hydrolysate. Each of the standard solutions contained the same quantity of arabinose per unit volume. The quantity of hydrolysate spotted was adjusted until it furnished a spot which contained the same quantity of arabinose as that of the standard solutions. By comparison of the size of the glucose spot resulting from the Mannich hydrolysate with the size of the spots from the standards, the ratio of glucose to arabinose was shown to be 1:1.

DISCUSSION

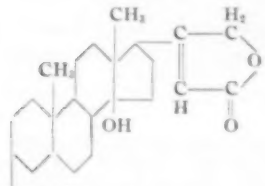
Euatroside, being hydrolyzed by both emulsin and strophanthobiase, shows the characteristic of a β-glucoside. The β-glucosidases produce from euatroside, besides glucose, a secondary glycoside, euatromonoside. The only sugar resulting from acid hydrolysis of the latter is arabinose, identified by its color reaction with *p*-anisidine HCl and the R_f values obtained with three different solvent systems as well as by mixed chromatograms. Chromatographically, the aglycon part of euatroside and euatromonoside behaves exactly like digitoxigen (3). The yellow fluorescence produced with trichloroacetic acid is also identical to that produced with digitoxigen. In contrast, the aglycone from digoxin, which differs from digitoxigenin by one additional hydroxyl group, produces an intense blue fluorescent color and gives a different R_f value. By acid hydrolysis of digitoxin not only digitoxigenin but another non-sugar substance is formed. The

latter has an R_f value of about 0.85 with Solvent System 2 and evidently is the anhydrodigitoxigenin (7). Euatroside and euatromonoside behave in the same way as digitoxin in this respect giving rise to this same second spot. Recently, Tam and Rosselet (8) have shown that the aglycon of euonoside from *Euonymus europaea* L. is digitoxigenin.

The presence of one disaccharide plus two monosaccharides in the sugar fraction obtained from the



O-arabinose-β-glucose
Euatroside



O-arabinose
Euatromonoside

Mannich hydrolysis of euatroside (Table I) is strong evidence that the carbohydrate portion of euatroside contains only two sugars, one glucose plus one arabinose molecule. If more than one arabinose or one glucose molecule were present in euatroside, it would be expected that at least two disaccharides and one trisaccharide would be present in the hydrolysate as breakdown products of the glycoside. The result of the enzymatic hydrolysis of euatroside and the fact that glucose and arabinose occur in the ratio of 1:1 show that the disaccharide is arabinose-β-glucoside. Arabinose is attached directly to the aglycon. From the above findings, the structure of digitoxigenin arabinosido-β-glucoside suggests itself for euatroside, digitoxigenin arabinoside for euatromonoside. The occurrence of arabinose in a natural cardiac glycoside has not been reported previously.

SUMMARY AND CONCLUSIONS

1. The structure of a cardiac glycoside from *E. atropurpurea* has been investigated by use of microtechniques involving paper chromatography.

2. The name *euatroside* has been proposed for this new glycoside and the name *euatromonoside* is suggested for the secondary glycoside derived from it.

3. From the data obtained, the structure digitoxigenin arabinosido-β-glycoside is proposed for *euatroside* and digitoxigenin arabinoside for *euatromonoside*.

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The Quantitative Estimation of Ergotamine and Ergotoxine by Paper Chromatography*

By D. D. JONES, H. KATYAMA, and V. E. TYLER, Jr.

Under specified conditions the R'_f values of ergotamine and ergotoxine were found to increase in proportion to the quantity spotted on a paper chromatographic strip. This change in R'_f value is used as a means of quantitative estimation of these alkaloids.

EXISTING paper chromatographic techniques for quantitative estimation of the water-insoluble ergot alkaloids involve either comparison of the intensity of the fluorescent spots with a series of known standards or elution of the alkaloids from the paper strip and subsequent colorimetric evaluation (1). The accuracy of the former method is estimated to be ±20%, and considerable experience is necessary to achieve this level in actual practice. The latter procedure is obviously time-consuming and requires colorimetric apparatus.

* Received December 7, 1956 from the College of Pharmacy, University of Nebraska, Lincoln.

A recent investigation in this laboratory (2) revealed that when ergotamine or the ergotoxine group of alkaloids was chromatographed by the procedure of Brindle, *et al.* (3), the R'_f values increased with the quantity of the alkaloid applied to the strip. In this chromatographic system ergotoxine does not separate into its three known components and for practical purposes may be considered as an entity. The present study was undertaken to determine if the phenomenon observed could be utilized for the rapid microquantitative estimation of ergotamine and the ergotoxine group (ergotoxine).

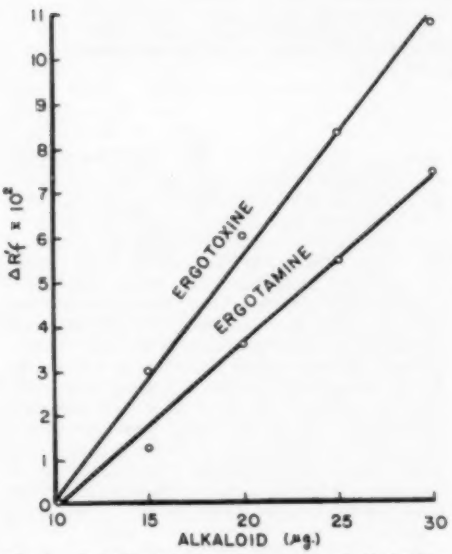

Fig. 1.—Relationship of the quantity of alkaloid on the strip to its $\Delta R'_f$ value.

EXPERIMENTAL

Chromatographic Method.—Strips of Whatman No. 1 filter paper, 1/2 in. × 20 in., were dipped in McIlvaine's buffer at pH 4.0 and allowed to air dry. Fresh alcoholic solutions of ergotoxine or ergotamine base, prepared from commercial samples of the alkaloidal salts[1] as described by Tyler and Schwarting (4), were spotted on the strips in 10, 15, 20, 25, and 30 μg. quantities. These quantities were spotted by the repeated application of appropriate small volumes, allowing each to dry completely before application of the next. The volume of a single application in no case exceeded 10 μl. The strips were placed in a saturated atmosphere of chloroform vapor in a glass chromatographic chamber and allowed to equilibrate for two hours. Descending formation, utilizing chloroform saturated with 0.1 *M* citric acid solution as the wash liquid, was then carried out for ninety minutes, the strips removed, dried, and the alkaloidal spots located by examination under ultraviolet light. R'_f values were calculated, based on the estimated front of the alkaloidal spot. Since the front of the spot was frequently somewhat u-shaped, the estimated front was taken as that point to which the substance would have migrated had the advance been uniform across the entire width of the strip.

Evaluation of the Strips.—It was observed that uncontrollable variations in the paper strips and slight temperature fluctuations had definite effects upon the reproducibility of the R'_f values. These effects were overcome to a large extent by the use of standard strips, spotted with 10 μg. of each of the alkaloids, which were chromatographed simultaneously with and utilized as a reference for higher concentrations. The change in R'_f value ($\Delta R'_f$) for higher concentrations of alkaloid was calculated from this reference R'_f value as follows:

$$\Delta R'_f = R'_{f_x} \text{ μg. alkaloid} - R'_{f_{10}} \text{ μg. alkaloid}$$

$\Delta R'_f$ plotted *versus* the quantity of each alkaloid spotted gave the linear relationships shown in Fig. 1. These relationships were the same for strips containing either alkaloid singly or a mixture of both ergotoxine and ergotamine.

Slight variations in R'_f values were encountered on different strips, and it was established that a minimum of four replications of each "unknown" concentration as well as the standard concentration were required to obtain results with an accuracy of ±2.5 – 3.0 μg. The accuracy could be improved considerably by increasing the number of replications. Each point on the standard curves in Fig. 1 represents an average of 24 strips from a total of six different determinations.

DISCUSSION

Since salts of the water-insoluble ergot alkaloids favor the stationary phase in this chromatographic system, it seems probable that large quantities of alkaloidal base tend to "overload" the salt-forming capacity of a given portion of the buffered paper strip and therefore increased quantities migrate a greater relative distance. This relative increase is, within limits, proportional to the amount of ergotoxine or ergotamine present and may be used as a means of quantitative estimation.

Because of certain recognized limitations of the paper chromatographic method, exact reproducibility of R'_f values is not possible. Figures based on R'_f values must be regarded, therefore, as approximations. Nevertheless, this method, which may be employed with great facility, is quite suitable for general laboratory use when extreme accuracy is not a requirement.

[1] Ergotamine tartrate supplied through the courtesy of Sandoz Pharmaceuticals.

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5-Hydroxytryptamine Antagonism by Lysergic Acid Diethylamide after Intracerebral Injection in Conscious Mice*

By THOMAS J. HALEY

The centrally induced effects of 5-HT and LSD and three of its derivatives have been studied following intracerebral injection in conscious mice. The symptoms observed after giving 5-HT were scratching and stupor. LSD and its derivatives caused hyperexcitability, piloerection, muscle incoordination, and sensitivity to touch. LSD also produced a sensitivity to sound, a muscle twitch in the lumbar area and a peculiar alternating stamping of the feet. LSD, 1-acetyl-LSD and 1-methyl-LSD were able to block the central effects of 5-HT whereas *d*-lysergic acid dimethylamide did not antagonize 5-HT.

AFTER DEMONSTRATING that parenteral injections of 5-hydroxytryptamine did not antagonize the effects of lysergic acid diethylamide in mice, Woolley and Shaw (1) suggested that intracerebral administration would be the method of choice for studying such antagonism. Gaddum and Vogt (2) reported that intraventricular injection of lysergic acid diethylamide antagonized the effects of 5-hydroxytryptamine in cats. However, this antagonism was nonspecific because other unrelated drugs were also effective. Since Haley (3) reported that intracerebral administration of 5-hydroxytryptamine and lysergic acid diethylamide produced opposite effects in conscious mice, the present investigation was undertaken to see if antagonism could be demonstrated in this species. Derivatives of lysergic acid were included in order to study their effects and possible antagonism of 5-hydroxytryptamine.

EXPERIMENTAL METHODS

Two hundred and fifty albino mice of both sexes, weighing 19–24 Gm. received intracerebral injections according to the method of Haley and McCormick (4). The effects of the injections were recorded merely by observing the changes in behavior of the mice and, unless specific percentages are given, mean that all mice responded for the stated time interval. Uninjected and saline-injected control animals were studied simultaneously with the medicated ones and the responses to sound, touch, the pinna reflex, ability to cling to a glass rod, and climb a slick glass plate set at an angle of 45°, were determined prior to and after injection. Groups of 20 mice were used for each dose although at times larger groups were used to ascertain the exact duration of a particular effect. The total doses were 30 μg. for 5-HT and 10 μg. for LSD and its derivatives. All doses are in terms of the salts. The LSD derivatives were studied by the blind technique. The drugs 5-HT, LSD, 1-acetyl-LSD 1-methyl LSD, and *d*-lysergic acid dimethylamide were dissolved in 0.9% saline and administered in a total volume of 0.05 ml. Control animals received similar injections of 0.9% saline. In the antagonism studies, the drugs were mixed prior to injection and given in a total volume of 0.05 ml. as a single injection. This was done to minimize the possibility of brain damage which occurs when two punctures of the brain are attempted. It is impossible to be certain that the same needle track would be entered on the second attempt.

RESULTS

5-HT.—Immediately after injection, a tachypnea of twenty-minutes' duration occurred. This was followed by a stupor of one and a half hours' duration. However, the mice could be aroused although they preferred to remain quiet One minute after injection, the mice scratched their faces. There was no change in sensitivity to sound or touch. The drug had no effect on the pinna reflex (ear twitch in response to touch) and there was no impairment in the ability of the animals to cling to a glass rod suspended 6 inches above the table. No differences, other than slowness in response, were observed in climbing the glass plate. The mice did not back up the plate, but climbed it in the same forward manner as the controls.

LSD.—After injection, a tachypnea of thirty-five minutes' duration was observed. Immediately after injection and during the next two minutes, 80% of the mice were aggressive and would bite a wooden stick or glass rod placed in front of them. Three to five minutes after injection, the mice developed a peculiar muscle twitch on the back in the pelvic area. This effect continued for thirty to thirty-five minutes during which time there were spasmodic movements of the tail or an alternating stamping of all four feet. Also the mice moved with short jerky steps. The twitch was absent during those

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intervals when the mice were walking or washing themselves. During the first fifteen minutes post-injection the mice were hyperexcitable. Both sound and touch would induce rapid running movements or jumping. Piloerection was observed ten minutes after injection and varied in duration from fifteen to sixty minutes with the majority of animals responding for the latter time interval. The pinna reflex was unaffected, but the muscle twitch and the hyperexcitability decreased the ability of the animals to cling to the glass rod during the first six minutes post-injection. The animals could climb the glass plate in the normal manner, but only with short jerky steps.

When 30 μg. of 5-HT and 10 μg. of LSD were given simultaneously only the effects of LSD were observed even though the period of observation was two and a quarter hours. Aggression was seen in 45% of the animals and the other LSD symptoms were of the same duration as with this drug alone. The decrease in aggressiveness was not considered significant because of difficulties encountered in observing it due to the short interval during which it occurred.

1-Acetyl LSD.—Immediately after injection a tachypnea of twenty-minutes' duration was observed. Within two minutes post-injection and during the next thirty minutes, 60% of the mice were aggressive and would bite. They appeared to be more aggressive than after LSD. During this same time interval the mice were sensitive to touch but not to sound. There was no muscle twitch as with LSD. Three minutes after injection the animals appeared sedated and although they could be aroused, they preferred to remain quiet during the next forty-five minutes. Piloerection began three to five minutes post-injection and lasted two and a half hours. Only a slight impairment was observed in the ability of the animals to cling to the glass rod. The pinna reflex was not affected. Ability to climb the glass plate also was not affected.

When 30 μg. of 5-HT and 10 μg. of 1-acetyl LSD were given simultaneously, 40% of the mice were aggressive and all were sensitive to touch and had a piloerection of the same duration as with the latter drug alone. Thus it was quite apparent that 1-acetyl LSD blocked the effects of 5-HT.

1-Methyl LSD.—There was an immediate tachypnea followed in three minutes by a bradypnea of short duration. During the first two to five minutes, 45% of the mice were aggressive and would bite. Hyperexcitability was observed three to five minutes post-injection and, although there was no sensitivity to sound, the animals were very sensitive to touch. Such stimulation resulted in jumping or rapid running movements. The mice could not climb the glass plate or cling to the glass rod for the first five minutes post-injection. All of these effects terminated within twenty minutes and no sedation was observed. Piloerection occurred within twenty minutes and lasted thirty minutes. The muscle twitch observed after LSD was not seen after this compound.

When 30 μg. of 5-HT and 10 μg. of 1-methyl LSD were given simultaneously, only 10% of the mice showed aggressive tendencies. The other effects, except for a slight sedation which developed after two minutes and lasted for three minutes, were the same as with the latter drug alone. Thus 5-HT was effectively blocked by the 1-methyl LSD.

***d*-Lysergic Acid Dimethylamide.**—Immediately after injection, a bradypnea of two minutes appeared and this was followed by a tachypnea of ten-minutes' duration. At no time were the mice aggressive. Hyperexcitability appeared two minutes post-injection and continued for about thirty minutes. Three to four minutes post-injection the mice became sensitive to touch and this state continued for six to nine minutes. This sensitivity resulted in rapid running movements and jumping which prevented the animals from climbing the glass plate or clinging to the glass rod for a period of five to eight minutes. No sensitivity to sound was observed. A piloerection of twenty-minutes' duration appeared two minutes post-injection. The pinna reflex was not affected and there was no evidence of a muscle twitch in the lumbar area. All the animals resumed their normal pre-injection activity twenty to twenty-five minutes post-injection.

When 30 μg. of 5-HT and 10 μg. of *d*-lysergic acid dimethylamide were given simultaneously there was an immediate tachypnea of twenty-minutes duration. After one minute the mice developed a hyperexcitability which lasted for thirty minutes. They became sensitive to touch four minutes post-injection and continued in this state for ten minutes. They were unable to climb the plate or cling to the rod for thirty minutes. A piloerection of twenty-minutes' duration also was seen. Eight minutes post-injection sporadic scratching of the external nares and face was observed. After forty-five minutes a stuporous state developed and lasted for two hours. In this experiment it appeared that not only was 5-HT not blocked but that each drug exerted its own particular effects.

DISCUSSION

The present results confirm and extend our previous observations regarding the pharmacological effects produced by LSD (3) in many but not all aspects, they agree with the report of Woolley (5). On the other hand, we have shown that the central effects of 5-HT can be evoked without the use of carbamylcholine or prior isolation of the mice. Furthermore, the effects produced by 5-HT in mice are in good agreement with those seen in cats by Feldberg and Sherwood (6) and Gaddum and Vogt (2). The antagonism between LSD and 5-HT in mice confirms the results of Gaddum and Vogt (1956) in cats. Thus the mouse appears to be as useful as the cat or dog for studying the pharmacological effects produced by the intracerebral injection of drugs. Chemical neutralization of 5-HT by LSD when the two drugs were mixed prior to administration does not appear likely for two reasons. There are no reactive groups on either molecule which could bring about such a neutralization. Furthermore, the inability of *d*-lysergic acid dimethylamide, which is identical in structure to LSD except for the substitution of methyl for ethyl groups, to block 5-HT indicates that chemical neutralization is not involved in 5-HT antagonism.

SUMMARY

The centrally induced effects of 5-HT and LSD and three of its derivatives have been

studied following intracerebral injection in conscious mice. The symptoms observed after giving 5-HT were scratching and stupor. LSD and its derivatives caused hyperexcitability, piloerection, muscle incoordination, and sensitivity to touch. LSD also produced a sensitivity to sound, a muscle twitch in the lumbar area, and a peculiar alternating stamping of the feet. LSD, 1-Acetyl-LSD and 1-Methyl-LSD were able to block the central effects of 5-HT whereas *d*-lysergic acid dimethylamide did not antagonize 5-HT.

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Isotonic Solutions V.*

The Permeability of Red Corpuscles to Various Salts

By CHARLES W. HARTMAN† and WILLIAM J. HUSA

In the present investigation the hemolytic method was used to obtain van't Hoff *i* values for various salts. It was thought that these data would be of value in the preparation of solutions isotonic with the blood, since data obtained by other methods are not always valid. A comparison of *i* values obtained by the hemolytic method, *i* values calculated from osmotic coefficients and activity coefficients, and *i* values calculated from freezing-point data is given.

THE HEMOLYTIC method has been used extensively in permeability studies but it has been used very little for obtaining data to be used in the preparation of isotonic solutions. Husa and Adams (1) showed that the hemolysis of red blood cells is not prevented by certain substances even though the substances may be present in concentrations which should be isotonic with the blood according to calculations based on colligative properties. Grosicki and Husa (2) determined *i* values for various substances quantitatively by using the hemolytic method. This method was also employed by Easterly and Husa (3) in studying the degree of hemolysis of rabbit and human erythrocytes in solutions of urea and urea derivatives.

In the present investigation the *i* values of various electrolytes were determined quantitatively by use of the hemolytic method. In order to compare these *i* values with those obtained from colligative property data, *i* values were calculated from the activity coefficients taken from Latimer (4), freezing-point data taken from the International Critical Tables (5), the osmotic coefficients of Robinson and Stokes (6), and freezing-point data from Lund (7). Hober (8) pointed out that in solutions of indifferent nonelectrolytes, erythrocytes undergo severe alterations which may be changed by the addition of a small proportion of an electrolyte such as sodium chloride. This has been further illustrated by (2) and (3). For this reason in this investigation experiments were conducted on two salts in the presence of 0.2 per cent sodium chloride in order to determine if the presence of sodium chloride would change the abnormal behavior of the salts.

The isotonic coefficient, van't Hoff *i*, is analogous to the osmotic coefficient introduced by Bjerrum (10) except that Bjerrum defines the osmotic coefficient, *g*, as the ratio of an observed colligative property to what the property would be if dissociation were complete. The equation which relates the osmotic coefficient to the van't Hoff *i* is

$$g = \frac{i}{\nu} \qquad (1)$$

where *g* is the osmotic coefficient and ν is the number of ions in a formula weight.

The following equation relates the van't Hoff *i* to the activity coefficient (11).

$$i = \nu + \nu/m \int_1^{\gamma} m\,d\ln\gamma \qquad (2)$$

ν is the number of ions for a formula weight, *m* is the molality, and γ is the activity coefficient.

* Received January 23, 1957, from the College of Pharmacy, University of Florida, Gainesville.
This paper is based on a dissertation presented to the Graduate Council of the University of Florida by Charles W. Hartman in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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TABLE I.—COMPARISON OF i VALUES OF SALTS, AT CONCENTRATIONS CAUSING 50 PER CENT HEMOLYSIS OF HUMAN ERYTHROCYTES, CALCULATED BY DIFFERENT METHODS FROM HEMOLYTIC DATA, FREEZING-POINT DATA AND ACTIVITY COEFFICIENTS

Salt	Blood Sample	i Value from Hemolytic Data[a]	i Value from I.C.T.F.P. Data[b]	i Value from Activity Coeff.[c]	i Value from Lund's F. P. Data
$CaCl_2$	37	2.77	2.62	2.57	...
$SrCl_2$	39	2.98	2.65	...	...
$MgCl_2$	39	2.94	2.68	...	...
$MgSO_4$	31	1.79	1.25	...	1.17
KBr	16	1.83	1.87	1.88	...
KCl	8	1.77	1.87	1.87	1.76
KF	52	1.71	1.87	1.83	...
KI	22	1.88	1.94	1.87	1.84
KNO_3	44	1.79	1.83	...	1.80
K_2SO_4	22	3.03	2.47	2.55	...
K_2SO_4[d]	56	2.71	2.60	2.80	...
NaBr	31	1.96	1.90	1.88	...
NaF	48	1.85	1.97	1.89	...
NaI	23	1.96	1.90	1.88	...
Na_2SO_4	48	3.21	2.50	2.49	2.51
Na_2SO_4[d]	56	2.76	2.61	2.52	2.62

[a] Hemolytic data from present investigation.
[b] International Critical Tables Freezing Point Data.
[c] Activity coefficients taken from Latimer.
[d] In the presence of 0.2 per cent NaCl.

If the i value is known at any concentration then the i value may be calculated for any concentration over a range for which accurate activity coefficients are known by the equation

$$m_1 i_1 - m_2 i_2 = \nu(m_1 - m_2) + \nu \int_{\gamma_2}^{\gamma_1} m d \ln \gamma \qquad (3)$$

The integral in Eq. 3 may be evaluated by plotting the negative natural log of the activity coefficient against the molality.

The i value at m_1 may be obtained from the osmotic coefficients of Robinson and Stokes (6). When the i value at m_1 is substituted into Eq. 3 and the integral is solved graphically then the i value at m_2 is obtained. If a series of such calculations is made, a curve may be prepared of the i values obtained and from this curve an i value for a salt at any concentration may be interpolated.

EXPERIMENTAL

Collection of Blood.—The blood used in the present investigation was obtained in exactly the same manner as described previously by Easterly and Husa (3). The only variation from the previous procedure was that human blood samples were obtained from many subjects.

Preparation of Solutions.—The solutions used in this investigation were prepared in the same manner as described by Easterly and Husa (3).

Quantitative Determination of Per Cent Hemolysis.—Details of the hemolytic method have been given by Grosicki and Husa (2).

Calculation of i Values.—In order to compare the i values obtained from the hemolytic data with i values obtained from measurement of colligative properties, the i value of sodium chloride was calculated from activity coefficients taken from Latimer (4) at concentrations causing 25, 50, and 75% hemolysis. Equation 2 was used to calculate the van't Hoff i from activity coefficients. The i value of sodium chloride at 0.1 m was calculated from the osmotic coefficients of Robinson and Stokes (6) by Eq. 1 and this value was used in Eq. 3. In Fig. 1 it will be seen that sodium chloride serves as a standard. When the concentration of sodium chloride and the concentration of the other substance are selected from the graph at a concentration causing the same per cent hemolysis the i value for the other substances is calculated by the osmotic equation used by Grosicki and Husa (2).

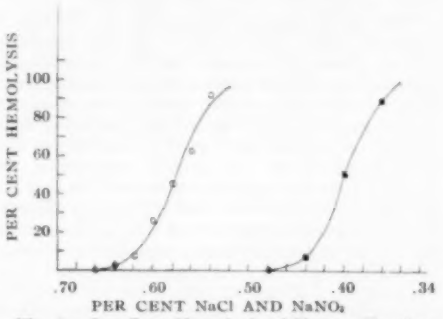

Fig. 1.—Per Cent Hemolysis of Human Erythrocytes in NaCl and $NaNO_3$ Solutions: O—O $NaNO_3$; ●—● NaCl.

DISCUSSION

Comparison of i Values.—In Table I it will be seen that the i values obtained by the hemolytic method using human blood, for salts with univalent anions and cations agree fairly closely with i values calculated from activity coefficients and freezing-point data. One salt that was an exception was potassium acetate which gave an i value of 1.58 with human blood and an i value of 1.52 with rabbit blood which is much lower than the i value calculated from freezing-point data. It was thought at first that pH caused the low value for potassium acetate solutions, however, in comparing this result with the

results of Grosicki and Husa (2) with sodium acetate and rabbit blood it appeared that the low value must be due to some cause other than pH. The above authors obtained an i value of 1.79 for sodium acetate using the hemolytic method on rabbit blood. It would seem that the pH effect would be the same in the case of both sodium and potassium acetate.

It will be noted in Table I that all of the salts used in this study which contain at least one divalent ion gave i values by the hemolytic method, using human blood, which were considerably higher than the i values calculated from activity coefficients and freezing-point data.

In Table I, it will also be noted that i values calculated from freezing-point data obtained from the International Critical Tables (5) agree fairly well with the i values calculated from the activity coefficients of Latimer (4), using the osmotic coefficients of Robinson and Stokes.

Effect of the Addition of Other Electrolytes.—In Table I it can be seen that the i value of sodium sulfate was lowered from 3.21, in experiments with sodium sulfate alone using human blood, to 2.80 when the solutions of sodium sulfate contained 0.2% sodium chloride. The i value obtained with potassium sulfate alone, using human blood, was 3.06 whereas in the presence of 0.2% of sodium chloride the i value obtained for potassium sulfate was lowered to 2.62. The results with sodium sulfate and potassium sulfate, in the presence of 0.2% sodium chloride, for rabbit blood were similar to the results obtained for human blood. Thus it would appear that the phenomena observed by previous workers (2, 7, 8) with nonelectrolytes is also true for electrolytes, *i. e.*, that the addition of small proportions of sodium chloride will alter abnormal effects of substances on the red blood cell.

Effect of Membrane Permeability on i Values.—Potassium salts gave i values by the hemolytic method which were slightly less than i values calculated from activity coefficients and freezing-point data whereas sodium salts, with the exception of sodium fluoride, gave i values slightly higher than i values calculated from activity coefficients and freezing-point data. A possible explanation for the low i values of potassium salts could be that the potassium salts penetrate the erythrocyte slightly, thereby increasing the internal osmotic pressure of the cell and at the same time decreasing the external osmotic pressure. Both of these factors would make the i value of potassium salts lower by increasing the concentration of potassium salt that must be in solution outside the cell to prevent hemolysis. This is in agreement with results of studies of Harris and Maizels (12).

The fact that the i values of the sodium salts were slightly higher than the values calculated from colligative property data may be due to a leakage of electrolytes from the cell. This would give the sodium salt an apparent i value higher than it would show if there were no electrolyte loss from within the cell. The high i values obtained for the salts containing at least one divalent cation may also be due to loss of electrolytes by the cell.

Difference in Results with Rabbit Blood and Human Blood.—It will be noted in Table II that the i value for magnesium sulfate obtained by the hemolytic method is 1.52, using rabbit blood, and 1.99 for human blood. It will be noted also that the i values listed for magnesium nitrate with human and rabbit blood have the reverse order of the i values listed for magnesium sulfate. It is of interest also that zinc sulfate gave an apparent i value of 921 with rabbit blood as compared to 541 with human blood. Zinc sulfate appears to strengthen the membrane of the red cell in some manner making the cell more resistant to hemolysis. As little as 0.006% zinc sulfate partially protected human erythrocytes from hemolysis in hypotonic solutions. This action was more pronounced for rabbit erythrocytes. The application of permeability data obtained from the use of one species to permeability relations involving a different species is not always advisable as has been pointed out before (2, 8).

TABLE II.—COMPARISON OF i VALUES OF SALTS OBTAINED BY THE HEMOLYTIC METHOD USING RABBIT AND HUMAN BLOOD

Salts	i Values[a] from Rabbit Blood	i Values[a] from Human Blood
Calcium Chloride	2.85	2.76
Magnesium Chloride	2.97	2.90
Magnesium Nitrate	3.10	2.41
Magnesium Sulfate	1.52	1.99
Potassium Acetate	1.52	1.58
Potassium Bromide	1.81	1.79
Potassium Chloride	1.78	1.77
Potassium Fluoride	1.74	1.72
Potassium Iodide	1.90	1.85
Potassium Nitrate	1.83	1.77
Potassium Sulfate	2.89	3.06
Sodium Bromide	1.88	1.95
Sodium Fluoride	1.89	1.81
Sodium Iodide	1.92	1.93
Sodium Sulfate	3.20	3.19
Strontium Chloride	2.97	2.94
Zinc Sulfate	921	542

[a] These i values represent the average of two samples of blood.

SUMMARY

1. Isotonic coefficients, i values, have been determined for seventeen salts by use of the hemolytic method. The values were compared with i values calculated from activity coefficients and osmotic coefficients and from freezing-point data. Sodium salts gave higher i values by the hemolytic method than those calculated from colligative property data whereas potassium salts gave lower i values by the hemolytic method than those calculated from colligative property data. Salts with at least one divalent ion also gave higher i values by the hemolytic method than those calculated from colligative property data.

2. The effect on the i values obtained by the hemolytic method due to the addition of small proportions of sodium chloride was studied. It was found that the addition of small proportions of sodium chloride produced i values for the salts which agreed more closely with i values calculated from colligative property data.

3. Unusual results were obtained with zinc sulfate. Results of the hemolytic method using human blood gave an apparent i value for zinc sulfate of 541. This was thought to be due to some action of the zinc sulfate on the red cell membrane to make it more resistant to hemolysis.

4. A comparison is given of results with rabbit and human blood.

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Effect of Glutathione and Some of Its Components on the Response of *Escherichia Coli* to Penicillin*

By ROBERTSON PRATT and YOKO OKANO

Glutathione (GSH) has little effect on the growth of *Escherichia coli* ATCC 6522 in the chemically defined medium that was used, but when penicillin also is present favors the early onset of action of the antibiotic. Of the component moieties of GSH, in the absence of penicillin, glutamic acid appreciably stimulates and glycine slightly stimulates early growth, while cysteine markedly prolongs the lag phase. Glutamic acid nullifies the depressing effect of cysteine, but glycine does not. In the presence of penicillin, glutamic acid and glycine, singly or together, seem to favor earlier evidence of the growth-depressing effect of the antibiotic than is produced by penicillin alone. The effect of cysteine on the reaction of the organisms to penicillin is difficult to interpret, but there was no evidence for inhibition of penicillin, such as might have been expected from some of the reports in the literature. In the presence of penicillin, organisms respond quite differently to GSH and to an equivalent (concentrationwise) mixture of the component moieties of GSH.

EXPERIMENTAL EVIDENCE suggests involvement of glutathione (GSH) in the mechanism of action of penicillin on sensitive gram-positive bacteria (1) and on resistant gram-negative organisms (2), and supports an earlier contention based on consideration of molecular configurations (3). Cysteine, which may be considered a moiety of GSH, also has been suggested as a cellular constituent that may be important in determining the response of organisms to penicillin (4).

There is reason to believe that penicillin may affect the same general biochemical reaction chains in gram-negative bacteria as in gram-positive species, but that it may intervene at different links in the reaction chains in the two groups of organisms. Since the concentration required to elicit the inhibitory effects is much greater for the former organisms than for the latter, the gram-negative species are classed as resistant. Much of the experimental work supporting this view has been reviewed (5, 6). Quantitative measurements of the sulfhydryl content of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) and of *Escherichia coli* exposed to penicillin (7) lend support to the concept that penicillin affects reactions involving —SH⇆S-S equilibria in both species.

The present report, an extension of work published previously (2), is concerned with the effect of GSH and some of its moieties on the response of *Escherichia coli* ATCC 6522 to penicillin. When this strain of *E. coli*, in a chemically defined medium, is exposed to concentrations of penicillin ranging from 1.25×10^{-4}

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M (4.66 mcg./ml.) to 2.0×10^{-4} M (74.6 mcg./-ml.), there is an initial period of growth which is followed by a transient period of inhibition (2). The duration of the initial period of growth and the degree of inhibition and its duration vary with the concentration of antibiotic. The lowest concentration causes only slight depression of growth between the eighth and tenth hours of incubation, but with the highest concentration, after an initial growth period of about two hours, the absorbance decreases until the cultures become optically clear at the fifth hour and remain so for more than twenty-four hours, after which time growth resumes. Ultimately the experimental cultures attain the same absorbance as the controls not exposed to the antibiotic.

When the organisms are exposed simultaneously to a minimally inhibitory concentration of penicillin and to an equivalent (or higher) molar concentration of GSH the slight depressing action of the antibiotic is nullified. At higher concentrations of penicillin, up to 10^{-4} M (37.3 mcg./ml.), addition of GSH in concentrations from 0.1 to 100 times the concentration of antibiotic improves slightly the early growth of the test organism but the early benefit is short-lived, and soon the absorbance of the cultures begins to decline. The concentration of GSH (within the limits specified) relative to the concentration of penicillin has little effect on the rate of decline but does affect its duration and the duration of the subsequent secondary lag period before growth of the cultures (as indicated by absorbance) resumes. A low penicillin/GSH ratio favors prolonged depression.

In the present experiments the effects of glutathione on the response of *E. coli* to penicillin were compared with the effects of the several moieties of GSH.

EXPERIMENTAL

The experimental technique was the same as used previously (2). In brief, *Escherichia coli* ATCC 6522 was maintained by daily transfers in Anderson's chemically defined medium, as modified by Cowie, *et al.* (8). Inocula for experiments were taken from cultures that had been incubated eighteen hours at 37°. Organisms were centrifuged and washed three times and finally were resuspended in distilled water. The density of the final suspension was adjusted to a transmission of from 46 to 48%, as determined in Pyrex tubes (18 mm. I.D.) in a Lumetron, Model 402 EF equipped with a neutral filter. One milliliter of this suspension was inoculated into 14 ml. of medium in each culture tube, giving an initial optical density of 0.02 to 0.025 and an initial viable count of approximately 200,000,000 organisms per milliliter. All culture solutions were adjusted with phosphate buffer to pH 6.8. Cultures were incubated in inclined roller tubes rotating at a rate of 5 r.p.m.

Solutions of glutathione, amino acids, and of penicillin were sterilized by filtration through sintered glass. Solutions of the inorganic compounds were sterilized in an autoclave for twenty minutes at 15 pounds pressure and 126°. Crystalline potassium benzyl penicillin, assaying 1587 units per milligram, was used.

In all of the experiments the initial concentration of penicillin was 5×10^{-5} M (29.6 units or 18.64 mcg./ml.). The initial concentration of glutathione was 5×10^{-3} M. Thus the ratio of penicillin to GSH was 1:100. This ratio was chosen because it was the one that gave maximum effects in previous experiments. The separate amino acids were provided in concentrations which were calculated on the basis of the composition of GSH (see below).

RESULTS

Effects in the Absence of Penicillin.—Figure 1, drawn from the averaged data of three experiments run in duplicate, shows the results obtained when *E. coli* was exposed to GSH (0.005 M) or to glutamic acid, glycine, or cysteine at concentrations equivalent to those that would be provided by 0.005 M GSH if it were completely dissociated into these three moieties. Also shown are the data for organisms exposed to a mixture of the three amino aids.

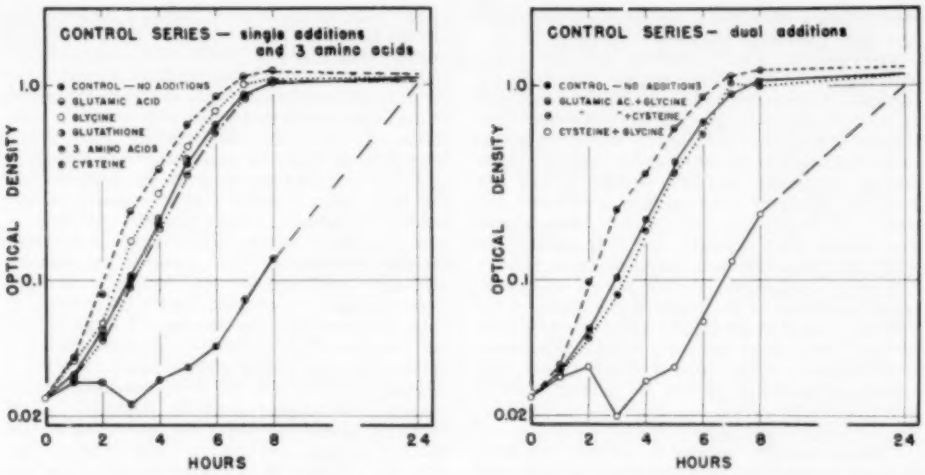
None of the treatments markedly affected the final population in the cultures, but addition of glutamic acid or of glycine to the culture medium improved the early growth of the organisms, largely by minimizing the lag period: L-cysteine, on the other hand, while not appreciably altering the final population, markedly retarded early growth by prolonging the lag period. When mixtures of the amino acids were used, curves for glutamic acid-glycine combinations were virtually indistinguishable from those for glutamic acid alone (compare Figs. 1 and 2). The presence of glutamic acid counteracted the depressing effect of cysteine, and there was little difference between the growth curves for control cultures and for those containing a glutamic acid-cysteine mixture (Fig. 2). Glycine did not significantly affect the response of the organisms to cysteine. When a mixture of the three amino acids was supplied, growth was, as might be expected from the foregoing observations, not significantly different from that in the control cultures or in those containing GSH.

The above observations on the individual compounds and on mixtures of them may be summarized, as far as their visible effects on growth of *E. coli* are concerned, as follows:

I Glutamic acid > glycine > GSH > control > cysteine;

II Glutamic acid-glycine > GSH > control > cysteine-glutamic ac. = glycine-glutamic ac.-cysteine > cysteine-glycine.

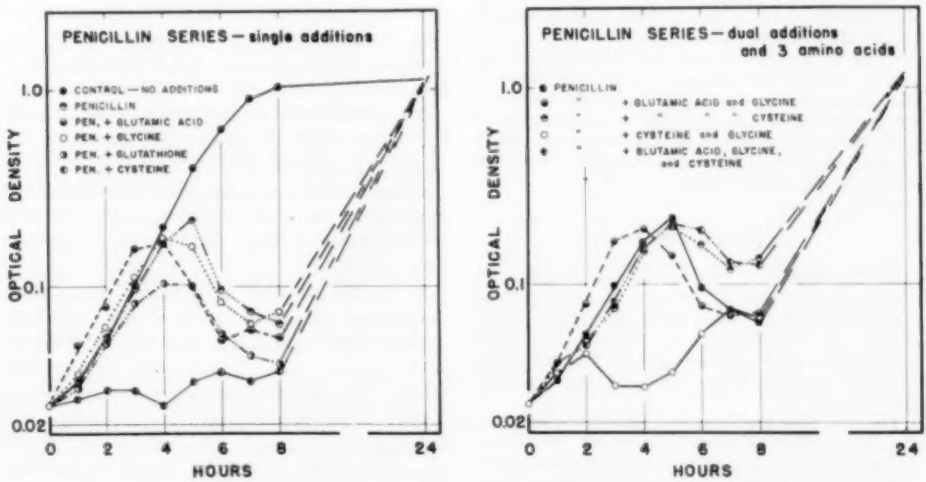
The inhibitory effect of cysteine is associated with the reduced form of the compound. Addition of cystine in equivalent molar concentration has no effect on growth of the organisms.



Figs. 1 and 2.—Response of *Escherichia coli* ATCC 6522 to additions of glutathione and its moieties in a synthetic medium lacking penicillin. Fig. 1 (*left*)—Single additions and mixture of glutamic acid, glycine, and cysteine. Fig. 2 (*right*)—Dual additions.

Effects in the Presence of Penicillin.—Interpretation of the effects of adding GSH or of its moieties on the growth of *E. coli* in the presence of penicillin is complicated by the penicillin-induced distortion of the growth curve. However, certain facts seem to stand out clearly. Addition of GSH, glutamic acid, or glycine to cultures containing penicillin does not affect the final population of the cultures, but it does affect the duration and the magnitude of the initial burst of growth that precedes the first visible effects of the action of penicillin. Of the three compounds, GSH is the most effective in inducing early onset of action of penicillin (Fig. 3). The absorbance of cultures exposed to GSH and penicillin, in contrast to those containing penicillin alone, does not reach such a high level during the initial period of growth, begins to decline sooner, and drops to a lower level.

In the presence of cysteine and penicillin there is no significant initial burst of growth (Fig. 3), and the lag phase is prolonged. However, the absence of an initial burst of growth is not surprising, since cysteine alone markedly prolongs the lag phase (Fig. 1). If one wished to throw caution to the winds and to attach significance to small changes, it might be concluded that the same trend is seen in the penicillin-cysteine curves as in the other penicillin-amino acid data, namely, that the first "burst" of growth in penicillin-cysteine cultures occurs between hours four and six and then is followed by a



Figs. 3 and 4.—Response of *Escherichia coli* ATCC 6522 to additions of glutathione and its moieties in a synthetic medium containing penicillin. Fig. 3 (*left*)—Single additions. Fig. 4 (*right*)—Multiple additions.

brief period of retardation. However, the available data do not afford strong support for this view.

The principal effect of glutamic acid and of glycine, separately (Fig. 3) or together (Fig. 4), in the presence of penicillin was to enable the organisms to grow more rapidly immediately following inoculation of the cultures, although the maximum optical density attained in the initial growth period was not affected markedly: however, whereas it took five hours for this level to be reached in penicillin cultures, only four hours were required when glycine was present with penicillin and only three hours when glutamic acid and penicillin were present. Following attainment of this initial maximum, organisms began to die, resulting in clearing of the solutions, and after six to eight hours (from the time of inoculation) there were only small differences in the absorbance of the cultures of this series. Following the period of decline, all cultures resumed growth and after twenty-four hours were as turbid as the controls containing no additions.

Growth was about the same for the first three hours in cultures containing penicillin alone, penicillin and GSH, or penicillin plus a mixture of the three amino acids. But at that point the similarity ceased. Whereas absorbance continued to increase for another two hours in the penicillin and in the penicillin-amino acid cultures, growth in the penicillin-GSH cultures was markedly retarded: the tubes suddenly began to clear, and after another three to five hours they were almost water-clear, the absorbance being almost as low as at the start of the experiment. After a few hours, however, these cultures resumed growth, and after twenty-four hours they had the same optical density as the controls.

Thus, in that the effect of penicillin is apparent sooner, is more pronounced, and lasts longer when GHS is present, GSH seems to favor the early action of penicillin against *E. coli*. The marked difference between the effect of GSH and of a (concentrationwise) equivalent mixture of the three amino acids is noteworthy. Whereas GSH seemed to favor the early onset of action of the antibiotic, the mixture of three amino acids partially antagonized its effects (Fig. 4).

Addition of glycine to cultures containing cysteine but no penicillin had comparatively little effect on the cysteine-induced prolongation of the lag phase (compare Figs. 1 and 2). But in the presence of penicillin, addition of glycine to cultures containing cysteine appreciably altered the shape of the growth curve (compare Figs. 3 and 4).

DISCUSSION

In the absence of analytical data on cell components and excretion products, it is difficult to evaluate the above observations in terms of mechanisms of penicillin action. Studies employing the Warburg technique have not been illuminating, and other approaches to the problem are now being investigated.

The fact that glutamic acid seems to nullify the depressing effect of cysteine does not, of course, necessarily imply that there is a direct antagonistic action of the former compound on the latter. The two compounds, whether present singly or together, may affect different rate-determining cell processes.

Since the organisms grow well in a medium containing glucose as the only organic component, it is evident that they can satisfy their amino acid requirements through biosynthesis. However, addition of glutamic acid stimulates their early growth in the simple synthetic medium that was used. Addition of GSH has comparatively little effect. Therefore, it might be argued that the depressing action of cysteine added singly to the cultures is not due to a direct depression of an essential cellular process but rather to a starving effect. In the presence of excess cysteine, there might be a tendency for the organisms to shunt most of the glutamic acid they synthesize into the formation of GSH. Thus, as rapidly as glutamic acid is biosynthesized, it may be combined into less readily utilized GSH and thus the effect of cysteine may essentially be to deprive the organisms of glutamic acid. If this hypothesis is correct, addition of surplus glutamic acid (beyond the amount normally produced by the bacteria) should minimize the growth-depressing effect of cysteine, and this was found to be the case (Fig. 2). It may be significant in this connection that glutamic acid counteracts the effects of cysteine more efficiently than α-ketoglutaric acid does (unpublished data). It is significant also that cysteine did not affect the growth of the organisms in broth cultures.

An interesting sidelight was noted in the cultures containing cysteine, whether penicillin was present or not. No evidence of growth occurred in any of the cysteine-containing cultures until evolution of H_2S was prominent, and when no H_2S was evolved, no growth occurred. These observations, which will be the subject of a later paper, seem pertinent in view of the recent report that H_2S may be an efficient source of —SH required for arylamine formation mediated by enzyme extracts of the E-26 strain of *E. coli* (9).

SUMMARY

1. The response of *Escherichia coli* ATCC 6522 to glutathione and to its component moieties, singly and in various combinations, was observed in the presence and in the absence of penicillin in a chemically defined culture medium.

2. In the absence of penicillin, glutamic acid stimulated early growth of the organisms appreciably, glycine less prominently, and L-cysteine, but not D-cysteine, markedly delayed the onset of the lag phase. Addition of glutathione or of mixtures of the three amino acids had little effect on growth.

3. In the presence of penicillin, glutathione accelerated the early onset of the inhibitory action of the antibiotic, mixtures of glutamic acid, glycine, and cysteine had an opposite effect. The effect of adding glutamic acid or glycine alone, in the presence of penicillin, was intermediate between the effects of adding glutathione and of adding a mixture of the three amino acids. Interpretation of the effects of adding cysteine in the presence of penicillin is

difficult because of the depressing effect that cysteine exerts, even in the absence of penicillin.

4. Some possible implications of the results are discussed.

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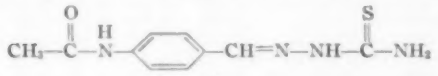
Amithiozone (Tibione) Analogs*

By DURWARD F. DODGEN, Jr.,† and W. LEWIS NOBLES‡

The preparation of thirty-seven 4-isobutylthiosemicarbazones to be tested for antitubercular activity is described. The majority of the compounds are variously substituted benzaldehyde, cinnamaldehyde, acetophenone, or benzalacetone derivatives. The nuclear substitutents include CH_3CONH; $C_2H_3O_2S$; CH_3O; i-C_3H_7; $(CH_3)_2N$; O_2N; H_2N; and Cl. Also, the isobutylthiosemicarbazones of a number of heterocyclic aldehydes and ketones are presented.

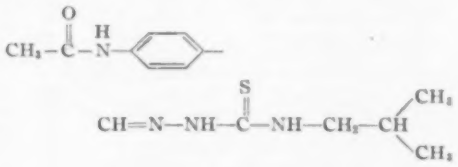
THE FIRST SYNTHETIC tuberculostat which approached the activity of streptomycin was *p*-acetamidobenzaldehyde thiosemicarbazone, otherwise known as amithiozone or Tibione (I). The activity of this compound was first announced by



I

Domagk and his associates (1) in 1946. Subsequently, extensive research seeking more active and less toxic compounds of this type has been reported in the literature (2). Most of these investigations have been concerned with the preparation and evaluation of analogs involving changes in the character and position of the substituents other than the thiosemicarbazone moiety in the molecule. Results in this area have thus far been disappointing since many compounds, while demonstrating marked activity *in vitro*, either were less active *in vivo* or so toxic that their clinical use was precluded.

Some earlier reports (3–5) had indicated that thiosemicarbazones which contained 4-alkyl substituents were less active than the unsubstituted thiosemicarbazones. More recently, however, Bernstein and his associates (6) demonstrated that *p*-acetamidobenzaldehyde - 4 - isobutylthiosemicarbazone (II) was more effective and less toxic than Tibione.



II

Thus, it appeared that alkyl substituted thiosemicarbazones had not received an adequate amount of systematic investigation.

Recently, we had reported the rather marked *in vitro* activity of the thiosemicarbazone of 4-(3-thienyl)-3-buten-2-one (7). The 4-isobutylthiosemicarbazone of this ketone was thus prepared and its antitubercular activity determined (8). The relative activities are reported in Table I. While this particular isobutylthiosemicarbazone did not demonstrate as marked activity as the parent thiosemicarbazone had, the fact that it did possess antitubercular activity stimulated further interest in this area. In an effort to determine if Bernstein's findings would be applicable to isobutyl derivatives of other analogs, a series of 4-isobutylthiosemicarbazone analogs of Tibione was prepared.

Other results (9) had indicated that the vinylogs of certain thiosemicarbazones were as active—if not more so—than the parent thiosemicarbazones. Consequently, we have also prepared the vinylogs of many of these 4-isobutylthiosemicarbazones in an attempt to further correlate the physiological activity of compounds and their vinylogs.

DISCUSSION

The 4-isobutylthiosemicarbazones prepared in the course of this work are reported in Table II.

4-Isobutylthiosemicarbazide was prepared from isobutylisothiocynate and hydrazine hydrate by

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TABLE I.—ANTITUBERCULAR ACTIVITY OF DERIVATIVES OF 4-(3-THIENYL)-3-BUTEN-2-ONE

		γ/ml. Causing Complete Inhibition →		Control
Organism	Strain	Thiosemicarbazone	4-Isobutylthiosemicarbazone	INH
M. tuberculosis	H37RV	0.16	20	0.024
M. tuberculosis	INH-Resistant	0.08	20	...
M. tuberculosis	Streptomycin-Resistant	0.08	20	...

the procedure of Pulvermacher (10); the isothiocyanate was obtained by following the method reported for the preparation of the methylisothiocyanate (11). In general, the carbonyl compounds were obtained commercially or prepared by the adaptation of methods reported in the literature.

p-Ethylsulfonylbenzaldehyde was obtained via a series of reactions which began with the interaction of sodium *p*-toluenesulfinate and ethyl iodide. Although Baldwin and Robinson (12) reported yields of 80–85% for the same type of reaction involving sodium benezenesulfinate, we were able to obtain only a 27% yield of the toluene analog. 4-Ethylsulfonyl toluene was converted to 4-(ethylsulfonyl)-benzaldehyde diacetate by means of chromic acid oxidation (13).

By modifying Nishimura's method for preparing *p*-nitrocinnamaldehyde (14), *p*-ethylsulfonylcin-

TABLE II.—4-ISOBUTYL THIOSEMICARBAZONES

No.	Carbonyl Compound	Yield, %	M.p.,[b] °C.	Formula	Analyses[a] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	*p*-Acetamidobenzaldehyde	84	159–161[c]	$C_{14}H_{20}N_4OS$	...	...	...	...
2	*p*-Acetamidocinnamaldehyde	25	205–208	$C_{16}H_{22}N_4OS$	60.35	60.86	6.99	7.13
3	Benzaldehyde	87	130–131[d]	$C_{12}H_{17}N_3S$	...	...	...	...
4	Cinnamaldehyde	63	178–179	$C_{14}H_{19}N_3S$	64.33	64.17	7.32	7.19
5	*p*-Ethylsulfonylbenzaldehyde	86	204–205[e]	$C_{14}H_{21}N_3O_2S_2$	...	...	...	...
6	*p*-Ethylsulfonylcinnamaldehyde[f]	54	169–172	$C_{16}H_{23}N_3O_2S_2$	54.31	53.92	6.53	6.37
7	*p*-Methoxybenzaldehyde	80	140–141[f]	$C_{12}H_{19}N_3OS$	...	...	...	...
8	*p*-Methoxycinnamaldehyde[f]	26	168–170	$C_{14}H_{21}N_3OS$	61.62	61.16	7.26	7.08
9	*p*-Isopropylbenzaldehyde	58	130–131[g]	$C_{15}H_{23}N_3S$	...	...	...	...
10	*p*-Isopropylcinnamaldehyde	51	137–138	$C_{17}H_{25}N_3S$	67.28	66.92	8.34	8.12
11	*p*-Dimethylaminobenzaldehyde	84	175–176[h]	$C_{14}H_{22}N_4S$	...	...	...	...
12	*p*-Dimethylaminocinnamaldehyde	58	215–217	$C_{16}H_{24}N_4S$	63.11	62.78	7.91	7.69
13	*p*-Nitrobenzaldehyde	89	198–199	$C_{12}H_{16}N_4O_2S$	51.40	51.63	5.75	5.79
14	*p*-Nitrocinnamaldehyde	41	208–210	$C_{14}H_{18}N_4O_2S$	54.88	55.11	5.92	5.69
15	2-Pyridyl aldehyde	84	148–151	$C_{11}H_{16}N_4S$	55.81	56.38	6.82	6.67
16	3-Pyridyl aldehyde	75	160–163	$C_{11}H_{16}N_4S$	55.81	55.69	6.82	6.53
17	4-Pyridyl aldehyde	80	164–165	$C_{11}H_{16}N_4S$	55.81	56.03	6.82	6.49
18	2-Thenaldehyde	91	150–151	$C_{10}H_{15}N_3S_2$	49.75	49.41	6.26	6.12
19	5-Nitro-2-thenaldehyde[f]	45	188–190	$C_{10}H_{14}N_4O_2S_2$	41.94	42.10	4.93	5.06
20	5-Nitro-2-furfural	81	179–181	$C_{10}H_{14}N_4O_3S$	44.43	44.54	5.22	5.16
21	5-Nitro-2-furan acrolein	89	176–178	$C_{12}H_{16}N_4O_3S$	48.63	48.66	5.48	5.45
22	Benzalacetone	77	125–126	$C_{15}H_{21}N_3S$	65.41	64.96	7.68	7.38
23	*p*-Acetamidobenzalacetone	71	114–115	$C_{17}H_{24}N_4OS$	61.41	61.27	7.28	7.16
24	*p*-Dimethylaminobenzalacetone	43	141–143	$C_{17}H_{26}N_4S$	64.11	63.93	8.23	8.09
25	*p*-Aminoacetophenone	67	166–167	$C_{13}H_{20}N_4S$	59.04	58.87	7.62	7.54
26	*p*-Chloroacetophenone	71	125–126	$C_{13}H_{18}ClN_3S$	55.01	54.83	6.39	6.26
27	*p*-Chlorobenzalacetone	67	137–138	$C_{15}H_{20}ClN_3S$	58.14	57.69	6.51	6.38
28	*p*-Methoxyacetophenone	51	113–114	$C_{14}H_{21}N_3OS$	60.19	59.82	7.58	7.68
29	*p*-Methoxybenzalacetone	41	99–100	$C_{16}H_{23}N_3OS$	62.90	62.76	7.58	7.47
30	*p*-Nitroacetophenone	86	163–164	$C_{13}H_{18}N_4O_2S$	53.02	53.19	6.16	6.21
31	*p*-Nitrobenzalacetone	51	169–170	$C_{15}H_{20}N_3O_2S$	56.23	55.98	6.30	6.28
32	2-Acetylthiophene	70	121–123	$C_{11}H_{17}N_3S_2$	51.73	51.68	6.71	6.64
33	2-Thenalacetone	86	115–116	$C_{13}H_{19}N_3S_2$	55.47	56.09	6.80	6.96
34	5-Chloro-2-acetylthiophene[k]	82	162–163	$C_{11}H_{16}ClN_3S_2$	45.40	45.73	5.55	5.62
35	3-Thenalacetone[f]	15	101–103	$C_{13}H_{19}N_3S_2$	55.47	55.78	6.80	6.63
36	5-Nitro-2-acetylfuran	86	176–177	$C_{11}H_{16}N_4O_3S$	46.45	46.27	5.67	5.53
37	2-Furfuralacetone	34	103–106	$C_{12}H_{19}N_3OS$	58.85	58.96	7.22	7.31

[a] Carbon and hydrogen analyses by Drs. G. Weiler and F. Strauss, Oxford, England.
[b] All melting points are uncorrected.
[c] Bernstein, J., *et al.*, *J. Am. Chem. Soc.*, **73**, 906(1951) report 151–152°.
[d] *Loc. cit.*, report 126–127°.
[e] *Loc. cit.*, report 197–198°.
[f] *Loc. cit.*, report 139–140°.
[g] *Loc. cit.*, report 129–130°.
[h] *Loc. cit.*, report 169–170°.
[i] According to method of Scholtz and Wiedemann, *Ber.*, **36**, 845(1903).
[j] According to the method of Patrick, T., and Emerson, W., *J. Am. Chem. Soc.*, **74**, 1356(1952).
[k] According to the method of Britton, S. B., and Nobles, W. L., THIS JOURNAL, **44**, 717(1955).
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namaldehyde was prepared from the corresponding benzaldehyde in a 21% yield. The essential change was an elevation of the reaction temperature.

p-Acetamidocinnamaldehyde was prepared according to Russell's procedure (15) as previously modified by others (16). *p*-Isopropylcinnamaldehyde was obtained by utilizing Thiele's procedure (17) for preparing *p*-tolylcinnamaldehyde. Nishimura's method (14) was used for preparing *p*-nitrocinnamaldehyde.

The method of Mohlau and Adams (18) was modified for the preparation of *p*-dimethylamino cinnamaldehyde. The essential change here was to terminate the condensation after approximately thirty minutes rather than the period of several hours used by the earlier workers. This tended to diminish the contamination of the product with higher condensation products.

We were not able to satisfactorily prepare *p*-chlorobenzalacetone following Lutz's procedure (19). However, by using a much larger excess of acetone and stopping the reaction after two hours, a 15–20% yield of *p*-chlorobenzalacetone was obtained. Utilizing this same method we were able to prepare *p*-acetamidobenzalacetone in a 27% yield.

EXPERIMENTAL

4-Isobutylthiosemicarbazones.—The method utilized was essentially that described by Campaigne, *et al.* (20), for thiosemicarbazones. Two-hundreths of a mole of the carbonyl compound was dissolved in 35 ml. of 50% ethanol (less soluble compounds required 75% ethanol). To this solution was then added five drops of glacial acetic acid and a solution of 2.94 Gm. (0.02 mole) of 4-isobutylthiosemicarbazide dissolved in 35 ml. of 50% ethanol. The mixture was then refluxed for approximately forty-five minutes. The crystalline residue, obtained on cooling, was collected and purified by recrystallization from dilute ethanol.

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The Sterilization of Plastic Intravenous Injection Equipment* by Ethylene Oxide Vapor†

By W. E. GRUNDY, E. J. RDZOK, W. J. REMO, H. E. SAGEN, and J. C. SYLVESTER

Intravenous injection equipment composed entirely of plastic is readily sterilized in the shipping carton by ethylene oxide vapor. Sterility was achieved in four hours but not in two hours with a temperature of about 75°F. and a gas pressure of 0.5 atmosphere. A technique for contaminating and for making quantitative measurements of organisms killed was developed. The basis of this method is a filter paper test strip impregnated with spores of *Bacillus globigii* A.T.C.C. 9372. The ethylene oxide vapor adsorbed by the plastic and the package had a marked sterilizing effect after the material was removed from the chamber. The experiments carried out in this study were designed to minimize this effect. Under practical conditions, the adsorbed Ethylene oxide provides an additional safety margin in the sterilization process.

THE BIOLOGICAL ACTIVITY of ethylene oxide was first recorded in 1938 by Cotton and Roark (2) who found it active against a variety of insect pests. Subsequently the antimicrobial activity of ethylene oxide was noted by Schrader and Bossert (13) in a patent granted in 1936. The early industrial uses of ethylene oxide were chiefly to reduce the microbial population of spices and other food products (5, 6). An excellent review of the uses and properties of ethylene oxide vapor as a sterilizing agent was pub-

* Abbott Laboratories' Trademark is Venoset.

† Received January 1, 1957, from Abbott Laboratories, Research Division, North Chicago, Ill.

lished by Phillips and Kaye in 1949 (11). The effectiveness and versatility of this material is now well established and industrial applications are becoming widespread (1). All types of microorganisms including viruses are readily killed (4, 9, 14). The remarkable penetrability of the vapor is an outstanding characteristic (10, 14, 15) and a variety of materials including plastics (3), antibiotics (7), hospital equipment (8), soil (15), and heat sensitive materials (1, 12, 14) can be sterilized with ethylene oxide. A recent review on sterilization by ethylene oxide by Sagen (12) points out the advantages and limitations of this process.

Ethylene oxide vapor offers two major advantages over heat treatment for the sterilization of intravenous injection equipment. It permits the use of materials that cannot be heat sterilized and the product may be sterilized in the package which reduces handling costs. This paper presents data on the ethylene oxide sterilization process as applied to plastic intravenous injection equipment and describes a technique for determining the effectiveness of this process.

EXPERIMENTAL PROCEDURE

The plastic injection equipment item on which these studies were made is pictured in Fig. 1. The Venosets were packed in individual open top trays which were cellophane wrapped. Twenty of these trays were packaged in a paper wrapped unit. Six of these paper wrapped units were packaged in a corrugated paperboard box. The sterilization experiments were performed with these corrugated boxes which contained 120 Venosets.

The exposure to ethylene oxide was carried out in a chamber which was first evacuated and then filled with the desired amount of ethylene oxide. In the preliminary experiments Carboxide[1] was employed but in subsequent studies undiluted ethylene oxide was used. The time of the exposure to the gas, the temperature, and the pressure were recorded for each run.

Since the plastic normally carries few microorganisms, it was necessary to develop a method for contaminating the Venosets with a known quantity of bacterial spores. *Bacillus globigii* ATCC 9372, which has been widely used as a test organism in ethylene oxide sterilization studies (11), was employed. The organism was grown in nutrient broth[2] in agitated 500-ml. Erlenmeyer flasks. The flasks contained 100 ml. of medium and were incubated at 32° C. for six days on a rotary shaking machine with an offset of 2¼ in. and a speed of 240 to 260 r. p. m. The spores were centrifuged, washed twice with water, and resuspended in 5% sterile lactose solution. The spore preparation was pasteurized at 80° C. for twenty minutes and lyophilized. The viable spore count of the dried material was determined by replicate agar plate counts. The dry spore preparations were very stable and one lot was used throughout these studies.

[1] A commercially available material which contains 90% carbon dioxide and 10% ethylene oxide.

[2] Difco peptone 10 Gm., beef extract 3 Gm., NaCl 5 Gm., and distilled water one liter, no pH adjustment.

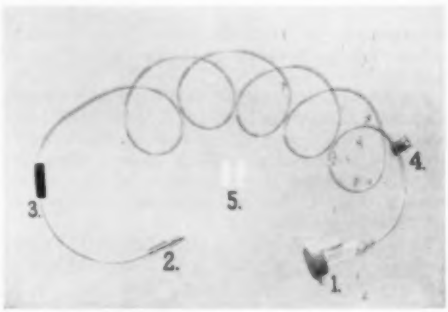

Figure 1.—The all plastic injection equipment (Venoset). 1—Cap assembly, 2—Needle adaptor and hood, 3—Injection site, 4—Adjustable pinch clamp, 5—Filter paper test strips carrying spores of *Bacillus globigii*.

Filter paper strips impregnated with a known number of *Bacillus globigii* spores were used to contaminate the sets. These were prepared by cutting filter paper of the type used for paper chromatography[3] into one-inch lengths. The strips of filter paper were moistened individually with 0.02 ml. of a suspension of *Bacillus globigii* spores containing 1,000,000 spores per ml. After several hundred strips were impregnated, they were dried in a 37° C. incubator for 24 hours. During the moistening and drying processes the paper strips were arranged cartwheel fashion in petri dishes. After the strips were dried, they were stored in tightly sealed vials until used. Sterilization of the paper before using it for test strips proved unnecessary.

The spore count of the strips was checked before each experiment by the following procedure. Ten strips were placed in a Waring Blendor with 100 ml. of sterile water and disintegrated for two to three minutes. Plate counts were made of this filter paper suspension using trypticase soy broth[4] solidified with agar and incubation at 30° C. The trypticase soy broth proved to be an excellent medium to bring out the deep orange pigment of *Bacillus globigii*. It was found that only 50 to 75% of the spores placed on the test strip were recoverable by this procedure. For this reason, the strips used in the various experiments were prepared by adding 20,000 spores to the strips in order to obtain 10,000 to 15,000 recoverable spores. Test strips prepared in the manner just described were found to give stable plate counts for at least six months.

To contaminate the Venosets for the various sterilization experiments the *B. globigii* filter paper strips were placed inside of the Venosets in the desired location prior to processing. After treatment with ethylene oxide gas the test strips were removed from ten sets, combined, and broken up in the Waring Blendor. Plate counts on trypticase soy broth agar and sterility tests with fluid thioglycollate medium were made on the filter paper suspension. Usually 20 ml. of the 100 ml. of suspension were employed for the sterility tests.

[3] Chromatography No. 613 9/16 in. wide. The Eaton-Dikeman Co., Mount Holly Springs, Pa.

[4] Baltimore Biological Laboratory, Baltimore 18, Md.

TABLE I.—STERILIZING EFFECT OF RESIDUAL ETHYLENE OXIDE VAPOR

Process Time, Hr.	Test Strips Cultured Immediately: Plate Count Spores/Strip	Test Strips Cultured Immediately: Sterility Tests	Test Strips Held in Package for 24 Hr.: Plate Count, Spores/Strip	Test Strips Held in Package for 24 Hr.: Sterility Tests
1/2	2,510	+	0	−
1	1,030	+	0	−
2	143	+	0	−
0 (control)	14,130	+	0	−

+ = contaminated; − = sterile; 0 = no organisms recovered.

Sterility tests were also performed on a number of uncontaminated Venosets that had been subjected to the same sterilization procedure as the contaminated sets. This was done by passing sterile water through the Venoset, manipulating of the plastic to force water in contact with all parts, and draining the water into large test tubes containing 100 ml. of fluid thioglycollate medium. The volume of sterile water which flowed through the Venoset was approximately 10 ml. The sterility test medium was incubated at 32–35° C. for at least one week.

Experiments were carried out employing different periods of exposure to ethylene oxide in order to determine the rate of killing of the spores under the experimental conditions.

RESULTS AND DISCUSSION

Early in our work with the sterilization of plastic materials it became apparent that ethylene oxide gas adsorbed by the plastic and the packages as a whole exerted a sterilizing effect after the materials were removed from the gas chamber. Data from an experiment with Carboxide are presented in Table I to illustrate this point. The Venosets used in this work were a standard design which were customarily sterilized by steam under pressure. The test strip containing the *B. globigii* spores was placed inside the sight glass. A total of 20 Venosets were cultured for each sterilization time. Ten were opened as soon as possible after sterilization and the test strips cultured. The other ten were allowed to remain in the unopened package of twenty for twenty-four hours before testing for viable spores. The results obtained in this experiment indicate that the residual ethylene oxide in the plastic and the package exerted a marked effect upon the viable spore count when the spore counts were delayed for twenty-four hours after the exposure to ethylene oxide.

In the subsequent experiments the filter paper strips were removed from the sets and disintegrated as soon as possible after exposure of the sets to the experimental sterilization procedure.

The results of the different periods of exposure to undiluted ethylene oxide vapor are presented in Table II. One corrugated paperboard carton enclosing 120 Venosets (six packages of twenty) was treated at one time. The 40 Venosets in the two central packages were contaminated with test strips. The other sets in the carton were untreated. A test strip was placed in the cap assembly and another under the needle adaptor hood of each of the 40 treated Venosets. As soon as possible after treatment with the gas the packages were broken open and spore counts were made using 30 of the 40 Venosets. The test strips were handled in groups of ten and those from the cap assembly were cultured separately from those under the needle adaptor hood. The data reveals that two hours were required to achieve sterility under the conditions employed. The area of the needle adaptor hood proved more difficult to sterilize than the cap assembly.

Sterility tests were carried out on 20 of the uncontaminated Venosets from each carton. These data were of no assistance in evaluating the process time since all tests from the thirty minute exposure through the six hour exposure were sterile. However, the results do indicate that the use of data ob-

TABLE II.—STERILIZATION OF VENOSETS WITH ETHYLENE OXIDE VAPOR

	Exposure Time, Hr. 1/2	1	2	4	6
Temperature, ° F.	72	74	80	72	73
EtO_2 Gas Pressure in Atmosphere	0.67	0.73	0.70	0.67	0.63
	Plate Count, Spores/Strip				
Cap Assembly	337	0	0	0	0
	396	0	0	0	0
	297	0	0	0	0
Adaptor Hood	6,200	740	0	0	0
	4,300	576	0	0	0
	4,400	533	0	0	0
	Sterility Tests				
Cap Assembly	+	1 of 4 (+)	−	−	−
	+	−	−	−	−
	+	−	−	−	−
Adaptor Hood	+	+	−	−	−
	+	+	−	−	−
	+	+	−	−	−

Control 1, 11,850 spores/strip.
Control 2, 11,666 spores/strip.

+ = contaminated; − = sterile; 0 = no organisms detected.

TABLE III.—STERILIZATION OF VENOSETS WITH ETHYLENE OXIDE VAPOR

Process Time, Hr.	Temperature, °F.	EtO_2 Gas Pressure in Atmosphere	Plate Count, Spores/Strip		Sterility Test	
			Cap Assembly	Adaptor Hood	Cap Assembly	Adaptor Hood
2	73	0.50	0	293	−	+
			0	203	−	+
			0	300	−	+
4	78	0.50	0	0	−	−

+ = contaminated; − = sterile; 0 = no organisms detected.

tained with contaminated Venosets to establish the minimum process time provides a margin of safety.

The larger sterilization equipment which was subsequently set up for production-scale operation was designed to operate with a controlled pressure of ethylene oxide vapor of 0.5 atmosphere. Since the smaller experimental equipment used more ethylene oxide, 0.63 to 0.73 atmosphere of pressure, two additional experimental trials were made with a gas pressure of 0.5 atmosphere to duplicate plant conditions. The experiments were conducted in the same manner as before. A summary of these results is shown in Table III. The data indicates that under the prescribed conditions a sterilization time of four hours is adequate to achieve sterility in the Venoset. In actual production practice, however, a longer process time has been found more convenient and is currently in use. Two additional safety features pertain under production-scale operations. The Venosets normally carry a much lower degree of contamination than was used in these studies. Secondly, the marked sterilizing effect of the ethylene oxide adsorbed on the plastic and the package is operative in the plant process.

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The Preservation of Aqueous Preparations Containing Nonionic Surfactants I.*

Growth of Microorganisms in Solutions and Dispersions of Nonionic Surfactants

By MARTIN BARR and LINWOOD F. TICE†

Studies have shown that various microorganisms, including *Pseudomonas aeruginosa*, *Aspergillus niger*, *Penicillium notatum*, and *Monilia albicans* are capable of growing in solutions and dispersions of nonionic surfactants of the fatty acid ester type. It was observed that these organisms are capable of splitting the ester linkages of these surfactants. This is first evidenced visually by the appearance of free fatty acids in the inoculated surfactant solutions. Further study revealed that these test organisms produce esterases which split the fatty acid from the surfactant molecule. The presence of esterases was demonstrated using a tributyrin testing procedure. The liberation of free fatty acid from polyoxyethylene 20 sorbitan monostearate (Tween® 60) solutions is reported.

NONIONIC SURFACTANTS of the sorbitan partial ester type[1] and their polyoxyethylene derivatives have found wide and increasing use in various products. Because of their unique properties, they have found wide applications as emulsifiers, solubilizers, dispersants, detergents, and as spreading, wetting, and antifoaming agents.

Engler (1) and Bolle and Mirimanoff (2) have reported that certain nonionic surfactants promote the growth of molds. No study has as

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† The authors wish to thank Dr. Louis Gershenfeld and Dr. Bernard Witlin of the Department of Bacteriology, Philadelphia College of Pharmacy and Science, for their suggestions during this study.

Atlas Powder Company, Wilmington, Del.

TABLE I.—THE GROWTH OF MICROORGANISMS IN 1 AND 5% W/V SOLUTIONS AND DISPERSIONS OF NONIONIC SURFACTANTS OF THE SORBITAN PARTIAL ESTER AND POLYOXYETHYLENE ESTER TYPES

Surfactant	Control[a]	*Pseudomonas aeruginosa*	*Monilia albicans*	*Aspergillus niger*	*Penicillium notatum*
Polyoxyethylene 20 sorbitan monolaurate	..	+s	+s	+s	+s
Polyoxyethylene 20 sorbitan monopalmitate	..	+s	+s	+s	+s
Polyoxyethylene 20 sorbitan monostearate	..	+s	+s	+s	+s
Polyoxyethylene 20 sorbitan monooleate	..	+s	+s	+s	+s
Sorbitan monolaurate	..	+	+	+	+
Sorbitan monopalmitate	..	+	+	+	+
Sorbitan monostearate	..	+	+	+	+
Sorbitan monooleate	..	+	+	+	+
Polyoxyl 40 stearate	..	+s	+s	+s	+s

[a] No inoculum. + = growth of microorganisms observed. s = splitting of ester observed.

yet been reported on the growth of microorganisms other than molds in solutions and dispersions of nonionic surfactants of the fatty acid ester type. No mention has been made of the splitting of the molecules of the nonionic surfactants by microorganisms. Such a study, is reported in Part I of this paper. The effectiveness of various substances as preservatives in products containing these surfactants is reported in Part II.

EXPERIMENTAL

Growth of Microorganisms in Aqueous Solutions and Dispersions of Nonionic Surfactants.—The purpose of this study was to determine whether various microorganisms are capable of growing in aqueous solutions and dispersions of the nonionic surfactants of the sorbitan partial ester type. One and 5% w/v solutions or dispersions of the following nonionic surfactants were prepared and inoculated with the test organisms: polyoxyethylene 20 sorbitan monolaurate,[2] polyoxyethylene 20 sorbitan monopalmitate,[3] polyoxyethylene 20 sorbitan monostearate,[4] polyoxyethylene 20 sorbitan monooleate,[5] sorbitan monolaurate,[6] sorbitan monopalmitate,[7] sorbitan monostearate,[8] sorbitan monooleate,[9] and polyoxyl 40 stearate.[10] The latter substance is not of the sorbitan partial ester type but is a polyoxyethylene ester made by direct reaction of fatty acid with alkylene oxide. It was included in this study, however, as it is recognized in U. S. P. XV (3) and is the surfactant in Hydrophilic Ointment (4). To 10 ml. quantities of the sterilized solutions and dispersions, previously sterilized at 121.3° for twenty minutes and contained in test tubes, were added 0.1 ml. of a 24-hour thioglycollate broth suspension of *Pseudomonas aeruginosa*[11] or a 72-hour thioglycollate broth suspension of *Monilia albicans*,[12] *Aspergillus niger*,[13] and *Penicillium notatum*.[14] All inoculations were made in quadruplicate. Two tubes of each inoculated surfactant solution or dispersion were placed in an incubator (37°) and two similar tubes were stored at room temperature (25° ± 1°). All tubes were observed for a period of two months for evidence of growth. The results are expressed in Table I.

Examination of Table I reveals that the four microorganisms utilized in this study are capable of growing in all the surfactants employed. Growth was evidenced in the surfactant solutions and dispersions within several days after inoculation. It was noted that the microorganisms are capable of splitting the molecules of some of the surfactants, all of which are esters. The splitting of the ester was determined by visual examination of the inoculated solutions for free fatty acid formation. Thus, ester splitting was observed in solutions of the polyoxyethylene sorbitan fatty acid esters and polyoxyl 40 stearate by the appearance of insoluble lauric, palmitic, stearic, or oleic acid, depending on the acid present in the ester. Since the sorbitan partial fatty acid esters (Span products) are not soluble in water, it was not possible to observe visually ester splitting by the presence of insoluble fatty acids in these suspensions or dispersions.

Acid number determinations were carried out on the surfactant solutions and dispersions before and after sterilization and the determinations indicated no change in the free fatty acid concentrations. All sterile controls remained stable for the duration of the test period.

Experiments were carried out in which the number of organisms in the inoculum were reduced and, in others, where washed organisms were used as the inoculum. Results indicated that the organisms were capable of growing in the surfactant esters and splitting them. The rate of growth and ester splitting was reduced, however, when the number of organisms in the inoculum was reduced and when washed organisms were used as the inoculum.

A Quantitative Determination of the Splitting of Tributyrin as a Result of Esterase Activity.—Since the microorganisms employed in this study are known to produce esterases (5), it was decided to carry out an experiment in order to obtain further information on the role esterase production by microorganisms plays in the splitting of molecules of the surfactant. An experiment was, therefore, devised to measure quantitatively the free acid released from an ester, tributyrin, during organism growth.

The three organisms studied in this experiment

[2] Tween® 20; [3] Tween® 40; [4] Tween® 60; [5] Tween® 80; [6] Span® 20; [7] Span® 40; [8] Span® 60; [9] Span® 80; [10] Myrj® 52; registered trademark names of the Atlas Powder Company.

[11] *Pseudomonas aeruginosa*, isolated from a patient at the University of Pennsylvania Hospital.

[12] *Monilia albicans*, isolated from a patient at Children's Hospital, Philadelphia.

[13, 14] *Aspergillus niger* and *Penicillium notatum*, isolated from contaminated products in the laboratory of the Department of Pharmacy, Philadelphia College of Pharmacy and Science.

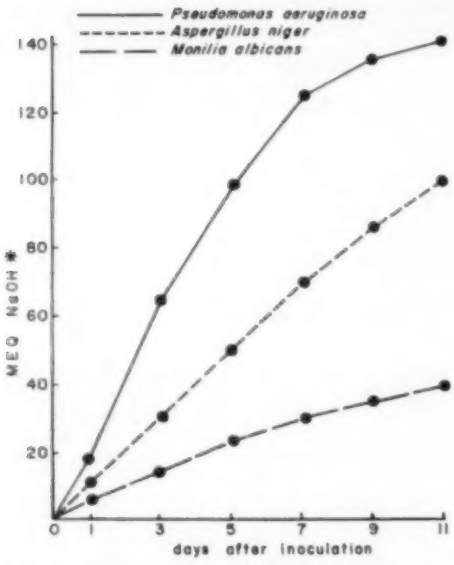

Fig. 1.—The esterase activity of three organisms on tributyrin.

* MEQ NaOH required to neutralize free acids due to the esterase activity of the organisms on 2 ml. of a 50% v/v tributyrin emulsion (1 ml. tributyrin).

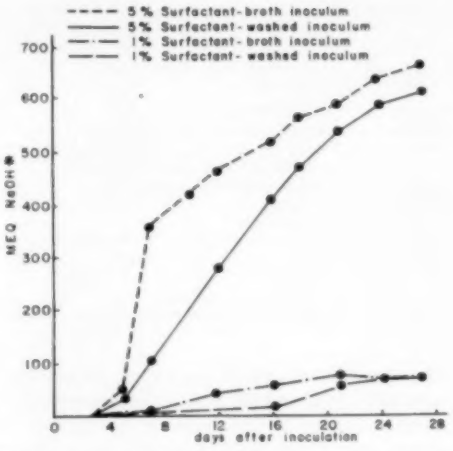

Fig. 2.—The splitting of polyoxyethylene 20 sorbitan monostearate (Tween® 60) by *Aspergillus niger*.

* MEQ NaOH required to neutralize the free acids due to the esterase activity of *Aspergillus niger* on 25 ml. surfactant solutions.

were *Pseudomonas aeruginosa*, *Monilia albicans*, and *Aspergillus niger*. The production of esterases by the strains of organisms employed was confirmed using the test method of Silver (5) which is based on Anderson's observation (6). The principle of the experiment was to titrate the free fatty acid liberated from a tributyrin emulsion which had been inoculated with the organisms under consideration. The procedure was as follows:

A 50% v/v emulsion of tributyrin was prepared using the following formula:

Tributyrin[18]	50%
Nutrient broth	25%
1% Methyl cellulose 4000	25%

The emulsion was prepared with the aid of a Waring Blendor. Two-milliliter quantities of this emulsion were transferred to test tubes and sterilized in an autoclave. Inocula consisting of 0.1 ml. of a 24-hour nutrient broth culture of *Pseudomonas aeruginosa* or a 72-hour nutrient broth culture of *Monilia albicans* and *Aspergillus niger* were placed in their respective tubes. One day after inoculation and on alternate days for a period of eleven days, each tube was titrated with 0.01091 *N* NaOH to a phenolphthalein end point after the addition of 10 ml. of neutralized alcohol. The milliequivalents of sodium hydroxide required to neutralize the fatty acid released from 1 ml. of tributyrin (contained in 2 ml. of a 50% v/v emulsion) were calculated and are reported in Fig. 1. Uninoculated controls were titrated with the alkali solution and it was found that 25.1 milliequivalents of sodium hydroxide were necessary to neutralize 2 ml. of the 50% tributyrin emulsion.

It is apparent from the examination of Fig. 1 that *Pseudomonas aeruginosa*, *Aspergillus niger*, and *Monilia albicans* are capable of producing esterases which result in the splitting of tributyrin. In this study, the bacterium, *Pseudomonas aeruginosa*, appeared to be a greater producer of esterase than molds. The faster initial rate of esterase production by bacteria is probably due to the fact that bacteria are faster initial growers than the molds—the greater the number of organisms, the greater the esterase production and, hence, the faster the ester is split.

The Splitting of Polyoxyethlene 20 Sorbitan Monostearate by Aspergillus niger.—In an effort to understand better the phenomena which occur when microorganisms are inoculated into surfactant solutions and dispersions, a study was carried out to determine quantitatively the splitting of polyoxyethylene 20 sorbitan monostearate by *Aspergillus niger*. It was decided to study quantitatively the effect of the test organism on 1 and 5% w/v aqueous solutions of the surfactant, both with and without other added nutrient. This was done in order to determine what roles the concentration of surfactant and other nutrient play in the splitting of the surfactant.

Twenty-five milliliters of 1 and 5% w/v sterile solutions of polyoxyethylene 20 sorbitan monostearate were placed in sterile 60-ml. bottles. One-tenth milliliter of a 72-hour thioglycollate broth inoculum of *Aspergillus niger* was added to both 1 and 5% w/v solutions of the surfactant and a similar procedure carried out using a washed inoculum of *Aspergillus niger;* i. e., free of thioglycollate medium. The bottles were placed in an incubator (37°) and the solutions were titrated at regular intervals with alcoholic 0.12 *N* NaOH to a phenolphthalein end

[18] The Matheson Company, Inc., East Rutherford, N. J.

point after the addition of 25 ml. of neutralized alcohol. The milliequivalents of sodium hydroxide necessary to neutralize the free fatty acid released in 25 ml. of the surfactant solutions due to the splitting of the surfactant by the organism are reported in Fig. 2. Uninoculated solutions of the surfactant were titrated with the alkali solution in a similar manner as a control. A total of 12 milliequivalents of sodium hydroxide was necessary to neutralize the 25 ml. of 1% w/v surfactant solution. A similar volume of a 5% w/v solution required 24 milliequivalents for neutralization. The amount of sodium hydroxide necessary for neutralization of the uninoculated surfactant control solutions remained constant during a 28-day period, showing that there was no hydrolysis of the ester within that time in the control.

From the data in Fig. 2, it is obvious that *Aspergillus niger* is capable of growing in solutions of polyoxyethylene 20 sorbitan monostearate and splitting the ester. The addition of a nutrient substance to the surfactant solution (such as the 0.1 ml. of thioglycollate broth in the broth inoculum) was found to stimulate organism growth. The faster the rate of growth, the more rapidly was the ester split.

DISCUSSION

It is quite evident that the microorganisms employed in this study are capable of growing in solutions of nonionic surfactants of the sorbitan partial ester and polyoxyethylene ester types. It is difficult to believe that microorganisms can utilize these surfactants for nutritive purposes directly, especially substances of the polyoxyethylene sorbitan partial ester type which have such large molecules. Studies are now in progress to ascertain what the nutrient is for the growth observed in the inoculated surfactant solutions.

The fact that the microorganisms employed in this study are capable of growing in surfactants and splitting their molecules becomes a real problem when one considers that these same microorganisms are among the most common contaminants in pharmaceutical products. The splitting of the surfactant to any extensive degree most probably would result in the destruction of its emulsifying and stabilizing properties and would likely result in the breakdown of the product. It is, therefore, necessary to add a preservative to products containing these surfactants in order to restrain the growth of microorganisms.

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The Preservation of Aqueous Preparations Containing Nonionic Surfactants II.*

Preservative Studies in Solutions and Products Containing Nonionic Surfactants

By MARTIN BARR and LINWOOD F. TICE†

Forty-eight substances and combinations were studied for their possible effectiveness as preservatives for solutions of nonionic surfactants of the sorbitan partial fatty acid ester and polyoxyethylene ester types. Results indicated that sorbic acid (0.2 per cent w/v); phenylmercuric borate (0.007 per cent w/v); phenylmercuric nitrate (0.01 per cent w/v); phenylmercuric acetate (0.007 per cent w/v); *n*-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide (Vancide® 89 RE, 0.04 per cent w/v); diethylene glycol monomethyl ether (methyl Carbitol, 3 per cent w/v); hexylene glycol (3 per cent w/v); and benzalkonium chloride (0.1 per cent w/v) are effective in preserving these solutions. The failure of various phenolic preservatives to preserve these surfactant solutions is attributed to complex formation between phenolic and polyether groups. Studies on various cosmetic formulations containing nonionic surfactants indicate that sorbic acid (0.2 per cent w/w) and combinations of sorbic acid and hexylene glycol are effective preservatives. These preservatives were also found effective in products containing sorbitol as well as nonionic surfactants.

IT WAS REPORTED in a previous paper (1) that microorganisms are capable of growing in solutions or dispersions of nonionic surfactants of the sorbitan partial fatty acid ester and polyoxyethylene ester types. It is, therefore, necessary to preserve products containing these surfactants in order to insure stability.

This study is concerned with an investigation of the effectiveness of various substances and

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TABLE I.—THE EFFECTIVENESS OF VARIOUS SUBSTANCES AS PRESERVATIVES FOR SOLUTIONS OF NONIONIC SURFACTANTS OF THE SORBITAN FATTY ACID AND POLYOXYETHYLENE ESTER TYPES*

Substance	% w/v	*Pseudomonas aeruginosa*	*Monilia albicans*	*Aspergillus niger*	Control**
Ethylparaben	0.10	+S.	+S	+S.	–
Butylparaben	0.05	+S.	+S.	+S.	–
Methylparaben	0.18	+S.	+S.	+S.	–
Propylparaben	0.15	+S.	+S.	+S.	–
Methylparaben Propylparaben	0.25 0.15	+S.	+S.	+S.	–
Methylparaben Propylparaben	0.18 0.02	+S.	+S.	+S.	–
Sodium benzoate	0.10	+S.	+S.	+S.	–
Sodium benzoate	0.50	+S.	+S.	+S.	–
Benzoic acid	0.20	+S.	+S.	+S.	–
Benzoic acid	0.40	+S.	+S.	+S.	–
Cetyl pyridinium chloride[a]	0.10	+	+	+	–
CTAB[b]	0.10	+	+	+	–
Benzalkonium chloride[c]	0.10	–	–	–	–
Hyamine 3104[d]	0.10	+	+	+	–
Hyamine 2389[e]	0.10	+	+	+	–
Hyamine 1622[f]	0.10	+	+	+	–
Hyamine 10-X[g]	0.10	+	+	+	–
Phenol	0.50	+S.	–	–	–
Cresol	0.50	+S.	–	–	–
Chlorothymol	0.40	+S.	+S.	+S.	–
Methylchlorothymol	0.20	+S.	–	+	–
Chlorobutanol	0.50	+S.	–	+	–
Chlorphenesin[h]	0.50	+S.	–	–	–
PCMX[i]	0.003	+S.	–	–	–
Hydroxymethyl furfural	0.50	+	+	+S.	–
Phenylmercuric borate	0.005	+	+	+	–
Phenylmercuric borate	0.007	–	–	–	–
Phenylmercuric nitrate	0.005	+	+	+	–
Phenylmercuric nitrate	0.01	–	–	–	–
Phenylmercuric acetate	0.005	+	+	+	–
Phenylmercuric acetate	0.007	–	–	–	–
Vancide® 89 RE[j]	0.04	–	–	–	–
Sorbic acid[k]	0.05	+	–	+S.	–
Sorbic acid	0.10	–	–	–	–
Sorbic acid	0.15	–	–	–	–
Sorbic acid	0.20	–	–	–	–
Butylhydroxy anisole[l]	0.05	+S.	+S.	+S.	–
Butylhydroxy anisole	0.10	+S.	+S.	+S.	–
Carbitol Solvent[m]	3	+	–	+	–
Carbitol Solvent	5	+	–	+	–
Cellosolve Solvent[n]	3	+	–	+	–
Cellosolve Solvent	5	+	–	+	–
Methyl Carbitol[o]	3	–	–	–	–
Methyl Carbitol	5	–	–	–	–

(*Continued on page 447*)

their combinations in preserving solutions and products containing these surfactants against the growth of microorganisms.

EXPERIMENTAL

Screening of Substances for Effectiveness in Preserving Nonionic Surfactant Solutions.—Forty-eight substances and combinations were studied for their effectiveness as preservatives for solutions containing nonionic surfactants of the sorbitan partial fatty acid and polyoxyethylene ester types. In the initial testing procedure, tests were carried out on solutions of surfactants known by previous work to support the growth of organisms (1). It soon became evident that the results obtained were similar regardless of the surfactant employed. It was also observed that the growth of organisms and the splitting of the surfactant molecule previously described (1) took place more quickly in 5% w/v surfactant solutions than in 1% w/v solutions. Therefore, all preservative studies were carried out on 5% w/v aqueous solutions of polyoxyethylene 20 sorbitan monostearate.[1] If satisfactory preservative activity was observed, repeat studies were performed on 1% w/v and 5% w/v aqueous solutions of a series of nonionic surfactants of the sorbitan partial fatty acid and polyoxyethylene ester types.

The testing procedure used was as follows: Aqueous solutions containing 5% w/v of polyoxyethylene 20 sorbitan monostearate were prepared. The individual substances and combinations to be studied for their effectiveness as preservatives were

[1] Tween® 60, Atlas Powder Company.

TABLE I—(*Continued*)

Substance	% w/v	*Pseudomonas aeruginosa*	*Monilia albicans*	*Aspergillus niger*	Control**
Butyl Carbitol[p]	3	+	+	+	−
Butyl Carbitol	5	+	+	+	−
Diethyl Carbitol[q]	3	−	−	+	−
Diethyl Carbitol	5	−	−	+	−
Methyl Cellosolve[r]	3	+	−	+	−
Methyl Cellosolve	5	+	−	+	−
Butyl Cellosolve[s]	3	+	−	−	−
Butyl Cellosolve	5	+	−	−	−
Diethyl Cellosolve[t]	3	+S.	+	−	−
Diethyl Cellosolve	5	+S.	+	−	−
Cellosolve Acetate[u]	3	+	−	+	−
Cellosolve Acetate	5	+	−	+	−
Dioxane[v]	3	−	+	−	−
Dioxane	5	−	+	−	−
Diethylene glycol	3	+S.	+	+S.	−
Diethylene glycol	5	+S.	+	+S.	−
Propylene glycol	5	+S.	+	+S.	−
Polyethylene glycol 400	5	+S.	+	+S.	−
Methoxy polyethylene glycol 350	3	+S.	+	+S.	−
Methoxy polyethylene glycol 350	5	+S.	+	+S.	−
Hexylene glycol	3	−	−	−	−
Hexylene glycol	5	−	−	−	−
Phenylethyl alcohol	1	+S.	−	−	−
Phenylethyl alcohol	2.5	+S.	−	−	−
G-271[w]	1	+S.	+S.	+S.	−
G-251[x]	1	+S.	+S.	+S.	−
Sodium propionate	1	+S.	+S.	+S.	−
No preservative	−	+S.	+S.	+S.	−

* All studies were carried out on 5% w/v polyoxyethylene 20 sorbitan monostearate solutions. If preservation was effected repeat determinations were performed on other nonionic surfactant solutions of the sorbitan partial fatty acid and polyoxyethylene ester types.

** No inoculations were made in these solutions. + = growth of microorganisms observed; − = no growth of microorganisms observed; S. = splitting of ester observed.

[a] Ceepryn chloride, Wm. S. Merrell Company, Cincinnati, Ohio.
[b] Cetyltrimethylammonium bromide, Fine Chemicals Company, New York, N. Y.
[c] Winthrop Laboratories, New York, N. Y.
[d] Dimethyldidodecenylammonium chloride, Rohm and Haas, Philadelphia, Pa.
[e] Alkyl (C_9-C_{15}) tolylmethyltrimethylammonium chloride, Rohm and Haas.
[f] (Para-diisobutylphenoxyethoxyethyl)-dimethylbenzylammonium chloride monohydrate, Rohm and Haas.
[g] (Para-diisobutylcresoxyethoxyethyl)-dimethylbenzylammonium chloride monohydrate, Rohm and Haas.
[h] Para-chlorophenylglyceryl ether, Chemo Puro Manufacturing Corporation, New York, N. Y.
[i] Para-chloro-meta-xylenol, Ottawa Chemical Company, Toledo, Ohio.
[j] N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide, R. T. Vanderbilt Company, New York, N. Y.
[k] 2,4-hexadienoic acid; [m] diethylene glycol monoethyl ether; [n] ethylene glycol monoethyl ether; [o] diethylene glycol monomethyl ether; [p] diethylene glycol monobutyl ether; [q] diethylene glycol diethyl ether; [r] ethylene glycol monomethyl ether; [s] ethylene glycol monobutyl ether; [t] ethylene glycol diethyl ether; [u] ethylene glycol monoethyl ether acetate; [v] diethylene dioxide; registered trademark names of the Carbide and Carbon Chemicals Company, New York, N. Y.
[l] Eastman Chemical Products, Inc., Kingsport, Tennessee.
[w] N-soya-N-ethylmorpholinium ethosulfate; [x] N-cetyl-N-ethylmorpholinium ethosulfate; registered trademark names of the Atlas Powder Company.

dissolved in the surfactant solutions and the solutions then sterilized in the autoclave at 121.3° for twenty minutes. If the preservative was not thermostable, it was added aseptically to the sterile surfactant solution. To 10-ml. quantities of the sterile solutions contained in test tubes were added 0.1 ml. of a 24-hour thioglycollate broth suspension of *Pseudomonas aeruginosa*[3] or a 72-hour thioglycollate broth suspension of *Monilia albicans*[3] and *Aspergillus niger*.[4] These organisms were selected because they are common contaminants and because preliminary experiments have shown them to be among the most difficult to restrain in surfactant solutions. Four tubes of each surfactant-preservative combination were inoculated with each organism and placed in the incubator (37°). Each was observed for evidence of growth or ester splitting for a period of thirty days. If the preservative appeared to be effective, repeat tests were carried out using a similar testing procedure but with observations being made for ninety days. The results of these studies are reported in Table I.

Examination of Table I reveals that, of the 48 substances and combinations studied for possible effectiveness as preservatives, the following proved to be of value: sorbic acid (0.10–0.20% w/v), phenylmercuric borate (0.007% w/v), phenylmercuric nitrate (0.01% w/v), phenylmercuric acetate

[3] *Pseudomonas aeruginosa*, isolated from a patient at the University of Pennsylvania Hospital.
[4] *Monilia albicans*, isolated from a patient at Children's Hospital, Philadelphia.
[4] *Aspergillus niger*, isolated from contaminated products in the laboratory of the Department of Pharmacy, Philadelphia College of Pharmacy and Science.

(0.007% w/v), *n*-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide (Vancide® 89 RE, 0.04% w/v), diethylene glycol monomethyl ether (methyl Carbitol®, 3% w/v), hexylene glycol (3% w/v), and benzalkonium chloride (0.1% w/v).

It was quite surprising to find that the methylparaben-propylparaben combinations tried did not completely protect the surfactant solutions against either growth or ester splitting. Growth of microorganisms and the resultant splitting of the surfactant molecules was much greater in 5% w/v surfactant solutions than in 1% w/v solutions. Both growth and ester splitting took place more rapidly with *Pseudomonas aeruginosa* than with the other test organisms.

It is of interest to note from Table I that all substances containing phenolic groups were ineffective as preservatives for the surfactant solutions despite the fact that most of these phenolic substances are highly regarded as preservatives. It is believed that complex formation between the phenolic preservatives and the polyether structures of the surfactants may be responsible for the failure of the substances tested to protect the surfactant solutions. Analogous phenomena have been reported by Higuchi and Lach, using polyethylene glycols (2).

It was decided to include the class of compounds commonly referred to as the "glycol-ethers" and "polyalkylene glycols" in this study for possible preservative activity. These substances heretofore have not been used as preservatives, but it was thought that some of their chemical groupings might prove toxic to the test organisms and find possible application as preservatives containing surfactants. Of the several compounds tested, methyl Carbitol and hexylene glycol did prove of value.

Although the phenylmercuric salts proved effective in preserving the surfactant solutions, it is doubtful if they are of much practical importance because of the toxicity and incompatibilities of the mercury ion. Such substances are not looked upon with much favor by dermatologists or cosmetologists.

Benzalkonium chloride was the only cationic agent tested which showed some effectiveness in preserving the surfactant solutions. It is not completely clear why this substance was the only one of the cationics providing protection. Its chemical nature, however, also often limits its use in certain products.

Of all the substances which protected the surfactant solutions, sorbic acid appeared to be the most promising. Examination of Table I reveals that sorbic acid is highly effective in preserving the nonionic surfactant solutions in concentrations of 0.1–0.2% w/v. Some growth and a slight degree of ester splitting appeared in some of the surfactant solutions containing only 0.05% w/v sorbic acid. Solutions of the surfactants containing 0.1–0.2% w/v sorbic acid have a pH of about 3.8–4.1. It was noted that solutions containing the surfactants and sorbic acid develop a yellow color upon standing. This color change does not appear to influence the preservative action of sorbic acid. When sorbic acid is incorporated into suspensions, lotions, emulsions, and ointments, no color change in the product is observed.

Preservative Studies on Cosmetic Formulations.—Since sorbic acid appeared to hold the greatest promise as a preservative for surfactant solutions, it was decided to study its effectiveness in protecting various cosmetic products containing such surfactants. It was also decided to include hexylene glycol in this study as well as combinations of sorbic acid and hexylene glycol since this latter substance also proved effective in protecting the surfactant solutions.

The formulas of the cosmetic preparations[5] included in this study are outlined below:

Formula No. 301. O/W Cold Cream

	%
Mineral oil	50.0
Beeswax	7.0
Tween® 40[6]	2.0
G-1726[7]	8.0
Water	33.0

Formula No. 314. O/W Hair Dressing Lotion

Petrolatum	7.5
Mineral oil	37.5
Beeswax	2.0
G-1425[8]	4.5
Arlacel® 83[9]	2.0
Water	46.5

O/W Baby Lotion

Mineral oil	35.0
Lanolin	1.0
Cetyl alcohol	1.0
Span® 80[10]	2.1
Tween® 80[11]	4.9
Water	56.0

Formula No. 318. O/W Face Cream

Stearic acid	15.0
Isopropyl palmitate	1.0
Arlacel® 60[12]	2.0
Tween® 60[13]	1.5
Sorbo®[14]	3.0
Water	77.5

Formula No. 322. O/W Hand Lotion

Stearic acid	7.0
Lanolin	0.5
Arlacel® 80[15]	0.5
Tween® 60	2.5
Sorbo®	5.0
Water	84.5

Formula No. 327. O/W Antiperspirant Cream

Stearic acid	8.0
Beeswax	2.0
Myrj® 52[16]	6.0
G-2162[17]	3.5
Water	58.5
Aluminum chlor-hydroxide complex	22.0

[5] The formula numbers for each cosmetic formulation studied correspond to those which appear in "A Guide to Cosmetic and Pharmaceutical Formulation with Atlas Products," 1953.

[6] Tween® 40, polyoxyethylene 20 sorbitan monopalmitate; [7] G-1726, polyoxyethylene sorbitol beeswax derivative; [8] G-1425, polyoxyethylene sorbitol lanolin derivative; [9] Arlacel® 83, sorbitan sesquioleate; [10] Span® 80, sorbitan monooleate; [11] Tween® 80, polyoxyethylene 20 sorbitan monooleate; [12] Arlacel® 60, a light-colored sorbitan monostearate; [13] Tween® 60, polyoxyethylene 20 sorbitan monostearate; [14] Sorbo,® a 70% w/v aqueous solution of crystalline *d*-sorbitol; [15] Arlacel® 80, a light-colored sorbitan monooleate; [16] Myrj® 52, polyoxyl 40 stearate, U. S. P. XV; [17] G-2162, polyoxyethylene oxypropylene stearate;

could not grow on the aqueous material below. In a

It should also be stated that sorbic acid is more ef-

Formula No. 342. W/O Cold Cream

Beeswax	10.0
Mineral Oil	50.0
Arlacel® 83	1.0
Lanolin	3.1
Borax	0.7
Water	35.2

Formula No. 344. W/O Hair Dressing

Petrolatum	7.5
Mineral oil	37.5
Lanolin	3.0
Arlacel® C[18]	3.0
Beeswax	2.0
Zinc stearate	1.0
Borax	0.5
Water	45.5

[18] Arlacel® C, sorbitan sesquioleate, Atlas Powder Company.

Individual lots of each of these cosmetic formulas were preserved with the following substances to test their effectiveness under conditions of use: 0.2% w/w sorbic acid; 3.0% w/w hexylene glycol; 1.0% w/w hexylene glycol plus 0.2% w/w sorbic acid; and 3.0% w/w hexylene glycol plus 0.2% w/w sorbic acid.

Inocula of 0.1 ml. of a 24-hour thioglycollate broth suspension of *Pseudomonas aeruginosa* or a 72-hour thioglycollate broth suspension of *Monilia albicans* and *Aspergillus niger* were added to 100-gram quantities of each product contained in 4-ounce glass bottles. The inocula were inserted into the interior and on the surface of the preparations. All inoculations were made in duplicate. The inoculated samples were then observed for a period of four months for evidence of visible growth of microorganisms, the production of odors, and physical change which would make the cosmetic product unusable or unsatisfactory. The results are tabulated in Table II.

Examination of Table II reveals that all inoculated samples of Formulas No. 301, 314, 318, 322, and the baby lotion, which did not contain preservatives, supported the growth of organisms and growth was observed also in the uninoculated controls. All of these preparations are O/W emulsions.

Formula No. 327 is an O/W emulsion containing nonionic surfactants, but it did not support the growth of microorganisms. This might be expected as the preparation is an antiperspirant containing an aluminum complex and aluminum salts are well known to inhibit the growth of microorganisms.

Formulas No. 342 and 344 contain surfactants but did not support growth. These preparations are W/O emulsions and such systems in which oil is the continuous phase are not by their physical nature good media for the growth of microorganisms.

Although Formulas No. 301, 314, 318, 322, and the baby lotion supported growth in unpreserved samples, sorbic acid, hexylene glycol, and their combinations prevented such growth. These studies indicate that these substances are effective in preserving emulsion products containing nonionic surfactants of the sorbitan partial fatty acid and polyoxyethylene ester types.

Additional samples of the test formulas were also inoculated with molds collected from spoiled samples of cosmetic emulsions and representing chance contaminants. All of the unpreserved products supported the growth of these organisms but, in those containing sorbic acid, hexylene glycol, and their combinations, no growth was observed except in one. This preparation was Formula No. 322 in which occasional failures were observed. The organisms growing on this were isolated, cultured, and inoculated into unpreserved surfactant solutions and others containing sorbic acid. Sorbic acid effectively inhibited their growth. It is quite possible that, since the growth of organisms was observed on the surface of Formula No. 322, the explanation lies in the formula of this product. It contains a large excess of stearic acid which rises to the surface upon standing, forming a surface film and stratification into two layers took place. Organisms quite conceivably might grow on this surface film which

TABLE II.—A STUDY OF THE EFFECTIVENESS OF SORBIC ACID, HEXYLENE GLYCOL, AND THEIR COMBINATIONS IN PRESERVING COSMETIC PREPARATIONS

Cosmetic Formula No.	Preservative	Test Organism: *P. aeruginosa*	Test Organism: *M. albicans*	Test Organism: *Aspergillus sp.*	Control[a]
301	A	+	+	+	+
301	B	−	−	−	−
301	C	−	−	−	−
301	D	−	−	−	−
301	E	−	−	−	−
314	A	+	+	+	+
314	B	−	−	−	−
314	C	−	−	−	−
314	D	−	−	−	−
314	E	−	−	−	−
O/W Baby lotion	A	+	+	+	+
O/W Baby lotion	B	−	−	−	−
O/W Baby lotion	C	−	−	−	−
O/W Baby lotion	D	−	−	−	−
O/W Baby lotion	E	−	−	−	−
318	A	+	+	+	+
318	B	−	−	−	−
318	C	−	−	−	−
318	D	−	−	−	−
318	E	−	−	−	−
322	A	+	+	+	+
322	B	−	−	−	−
322	C	−	−	−	−
322	D	−	−	−	−
322	E	−	−	−	−
327	A	−	−	−	−
327	B	−	−	−	−
327	C	−	−	−	−
327	D	−	−	−	−
327	E	−	−	−	−
342	A	−	−	−	−
342	B	−	−	−	−
342	C	−	−	−	−
342	D	−	−	−	−
342	E	−	−	−	−
344	A	−	−	−	−
344	B	−	−	−	−
344	C	−	−	−	−
344	D	−	−	−	−
344	E	−	−	−	−

[a] No inoculation of organism; A = no preservative; B = 0.2% sorbic acid; C = 3% hexylene glycol; D = 1% hexylene glycol and 0.2% sorbic acid; E = 3% hexylene glycol and 0.2% sorbic acid; + = growth observed within 4 months; − = no growth observed within 4 months.

as preservatives for solutions containing non- sorbitan partial fatty acid ester and polyoxyethyl-

could not grow on the aqueous material below. In a better balanced formula this might not happen.

Since results indicate that the products containing 0.2% w/w sorbic acid alone were protected against the growth of microorganisms, it is believed that this substance is valuable as a preservative. Hexylene glycol and other substances previously found to be effective in the preservation of surfactant solutions may also find use as preservatives in certain products.

DISCUSSION

Examination of the results obtained from studies on the preservation of solutions and cosmetic products containing nonionic surfactants of the sorbitan partial fatty acid and polyoxyethylene ester types shows that 0.2% w/w sorbic acid was the most effective preservative found.

Sorbic acid is 2,4-hexadienoic acid (CH_3·CH:CH·CH:CH·COOH). It is a white crystalline solid, has a molecular weight of 112.12, melts at 134.5°, and decomposes at 228° (3, 4). It is soluble in water to the extent of 0.16% w/w at 23.3°, the solubility increasing to 0.22% w/w in a phosphate buffer at pH 4.4 and to 1.02% w/w in a similar buffer at pH 5.9 (5). The substance is more soluble in the presence of surfactants such as were employed in this study.

Gooding has reported that certain of the α,β-unsaturated fatty acids, including sorbic acid, are good fungistatic agents for foods and food wrappers (6). Sorbic acid has been found to be superior to sodium benzoate as a fungistatic agent in cheese, cheese products (7), and margarine (6). It is nontoxic (8) and has been found to be metabolized in the same manner as caproic and butyric acids, being completely oxidized to CO_2 and H_2O (9). Lehman and Patterson (10) of the Food and Drug Administration state that comprehensive pharmacological data have established the safety of sorbic acid for incorporation in films and other food packaging materials where the sorbic acid actually migrates into the food. It has also been stated that sorbic acid may be used with safety in pharmaceutical and cosmetic products (11).

As previously stated, Gooding reported that sorbic acid is an excellent fungistatic agent. His results are in agreement with the findings reported in this paper that sorbic acid is effective in protecting products against molds. Phillips and Mundt (12), Vaughn and his students (13–15), Lindberg, *et al.* (16), and Hansen and Appleman (17) found that sorbic acid is much more effective against all types of catalase-positive microorganisms tested than against the catalase-negative bacteria such as the lactic acid bacteria and clostridia. This agrees with the results reported wherein catalase-positive organisms such as *Staphylococcus aureus*,[19] *Pseudomonas aeruginosa*, and *Bacillus subtilis*[20] were found to be inhibited by sorbic acid. Catalase-negative organisms, such as the lactic acid organisms and clostridia, are not believed to be likely contaminants of products utilizing the nonionic surfactants, especially those which are external preparations.

[19] *Staphylococcus aureus*, FDA 209, A.T.C.C. No. 6538.
[20] *Bacillus subtilis*, isolated as an air contaminant from the Bacteriology Laboratory, Philadelphia College of Pharmacy and Science.

It should also be stated that sorbic acid is more effective in an acid medium; adjustment of pH on the acid side, therefore, might be expected to increase the effectiveness of this substance.

Melnick, Luckmann, and Gooding (18) have presented a mechanism for mold inhibition by sorbic acid based on Mukerjee's (19) observations of cyanide inhibition of butyric acid oxidation. One step in the growth of molds in the presence of fatty acids is dehydrogenation of the fatty acids to unsaturated compounds. This reaction, which is vital to the growth of the mold and even to its survival, is accomplished by a dehydrogenating enzyme system. The accumulation of the end products of this enzyme reaction (unsaturated fatty acids) as in any enzyme reaction will inhibit the reaction itself. More specifically, an excess quantity of sorbic acid in a product will inhibit the dehydrogenase enzyme system in the molds. This important enzyme system is essential for mold propagation and even survival (7, 20). Thus, with the introduction of a small mold population, a small concentration of sorbic acid added remains as such in a product. In the presence of a high mold population, the concentration of all the enzyme systems is increased with the result that sorbic acid may be metabolized with no inhibition of the dehydrogenase systems occurring. Under such conditions, mold growth will not be effectively restrained.

Sorbic acid has proved to be effective in inhibiting the growth of microorganisms in the solutions and cosmetic products included in this study with but a single exception. The conditions of the testing procedures were quite severe as heavy inocula were introduced in the samples; thus, a fairly high organism population was present in testing the effectiveness of sorbic acid. From the theory of the mechanism of sorbic acid's action, as already described, it is conceivable that, with gross contamination, a high enough organism population might be present in a product to permit the metabolism of the sorbic acid with the resultant spoilage of the product. Therefore, a maximum effort should be made to insure cleanliness and sanitation in making products to be preserved with this substance.

The cosmetic products and solutions containing the surfactants and preserved with sorbic acid have remained stable for a long period of time. At the time of the writing of this paper, the cosmetic products had been stored for four months. There was, as yet, no evidence that sorbic acid loses its preservative activity in products upon aging. Sorbic acid is oxidizable by air to form peroxides and aldehydic products. However, studies in packaged cheeses revealed no oxidative deterioration of the substance (21). Further studies are contemplated on the possibility of oxidation of sorbic acid in products upon long periods of standing. If such occurs, it may be necessary to include an antoxidant in such products.

It is also suggested that some of the other substances previously shown to be effective for preserving the nonionic surfactant solutions may be useful.

SUMMARY

1. Of the more than 50 substances and combinations studied for their possible effectiveness

as preservatives for solutions containing nonionic surfactants of the sorbitan partial fatty acid and polyoxyethylene ester types, the following were found to be effective: sorbic acid (0.10–0.20 per cent w/v), phenylmercuric borate (0.007 per cent w/v), phenylmercuric nitrate (0.01 per cent w/v), phenylmercuric acetate (0.007 per cent w/v), Vancide® 89 RE (0.04 per cent w/v), methyl Carbitol (3 per cent w/v), hexylene glycol (3 per cent w/v), and benzalkonium chloride (0.1 per cent w/v).

2. It is believed that the failure of phenolic substances to preserve the surfactant solutions is a result of complex formation between the phenolic groups and the polyether groups of the surfactants.

3. It was found that 0.2 per cent w/w sorbic acid, 3 per cent w/w hexylene glycol, a combination of 0.2 per cent w/w sorbic acid and 1 per cent w/w hexylene glycol, and a combination of 0.2 per cent w/w sorbic acid and 3 per cent w/w hexylene glycol are effective preservatives for products containing nonionic surfactants of the sorbitan partial fatty acid ester and polyoxyethylene ester types. An occasional failure in the preservation of one cosmetic product is reported. Some possible explanations for such failures are presented.

4. As a result of studies completed thus far, sorbic acid (0.2 per cent w/w) has been found to be the most satisfactory preservative for products containing nonionic surfactants of the sorbitan partial fatty acid ester and polyoxyethylene ester types.

5. The mechanism of the action of sorbic acid in inhibiting the growth of microorganisms, as reported by Melnick, Luckmann, and Gooding, is described and believed to be fully consistent with the results obtained in this investigation.

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A Note on the Variability of Alkaloid Content of Domestic Ergot*

By C. K. ATAL and A. E. SCHWARTING

ALKALOID VARIABILITY, qualitative and quantitative, has been reported for ergot of European origin. Alkaloid analysis of North American ergot has been reported for lot samples only. Individual sclerotia of four lots of domestic rye ergot were subjected to the following experimental procedure. A small portion of a sclerotia was aseptically transferred to a nutrient agar for growth. The remainder of the sclerotia (70–200 mg.) was analyzed (1) for total and water-soluble alkaloid content. Only those sclerotia which gave growth were analyzed.

These data, representing 110 determinations, together with other data not reported here, demonstrate the usual variability among field samples.

TABLE I.—ALKALOID CONTENT

Sample Origin	Total Alkaloid, % Highest	Lowest	Average	Water-soluble Alkaloid, % of Total Alkaloid
Canada	0.729	0.007	0.340	0.095
Connecticut	0.509	0.208	0.361	0.109
Minnesota	0.550	0.000	0.302	0.146
North Dakota	0.562	0.001	0.284	0.110

* Received October 26, 1956, from the School of Pharmacy, University of Connecticut, Storrs.

REFERENCE

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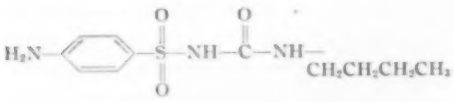
A Note on the Crystallography of Carbutamide (BZ-55)*

By HARRY A. ROSE and ANN VAN CAMP

CARBUTAMIDE (compound BZ-55) has been shown to have a hypoglycemic effect. Data are presented here which permit the identification of this compound by crystallographic methods.

The compound has the chemical name N'-(*n*-butyl carbamyl) sulfanilamide and has a structural formula as follows:



EXPERIMENTAL

Carbutamide was recrystallized from isopropanol-water solution and gave orthorhombic prisms with elongation parallel to the *c* axis.

The x-ray powder diffraction data were obtained using copper radiation and nickel filter with a camera 114.6 mm. in diameter. A wave-length value of 1.5405*A* was used in the calculations.

TABLE I.—X-RAY DIFFRACTION DATA

Unit cell dimensions, $a_0 = 10.00A$, $b_0 = 15.33A$, $c_0 = 9.28A$
Formula weights per cell, 4
Formula weight, 255.34
Density, 1.277 Gm./cc. (flotation), 1.269 Gm./cc. (x-ray)
Axial ratio, $a:b:c = 0.6523:1:0.6054$
Space group, $D_2^4 - P2_12_12_1$

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA

Refractive indices (5893*A*, 25°) $\alpha = 1.528$, $\beta = 1.544$, $\gamma = 1.700$
Optic axial angle, $(+)2V = 38°12'$ (calcd. from α, β, and γ)
Acute bisectrix, $\alpha = c$
Optic axial plane 100

* Received November 23, 1956, from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Ind.

The indexing of the powder pattern was done on the basis of single crystal rotation patterns around both the *b* and *c* axes.

On heating, the sample used for the diffraction data given here melted in the range 139–142° as determined on a Kofler hot stage. The melt did not crystallize on cooling.

TABLE III.—X-RAY POWDER DIFFRACTION DATA

d	I/I_1	*hkl*	*d* (calcd.)
8.42	0.67	110	8.38
7.89	0.67	011	7.94
		020	7.77
6.75	0.67	101	6.80
6.06	1.00	120	6.08
5.02	0.11	200	5.00
4.73	0.40	210	4.75
4.53	0.13b	002	4.64
		130	4.55
		012	4.44
4.21	0.67	102	4.21
4.05	1.00	112	4.06
3.82	0.05	040	3.83
3.69	0.27	122	3.69
3.57	0.27	140	3.58
3.36	0.27b	202	3.40
		141	3.34
		212	3.32
3.04	0.40	240	3.04
2.91	0.08b	150	2.93
		113	2.90
2.83	0.05	142	2.83
2.76	0.03	123	2.76
2.65	0.03b	312	2.67
		203	2.63
2.48	0.08	400	2.50
2.40	0.11		
2.27	0.11		
2.22	0.05b		
2.16	0.05		
2.11	0.05		
2.03	0.05		
1.979	0.03		
1.941	0.05		
1.897	0.05		
1.834	0.05		
1.787	0.03		
1.732	0.03		

BOOKS RECEIVED

Selected Scientific Papers from the Instituto Superiore di Sanita. Vol. 1, Part 1, 1957. Interscience Publishers, Inc., New York, 1957. 233 pp. 17 x 24 cm. Price $8.10.

A Glossary of Mycology. By WALTER H. SNELL and ESTER A. DICK. Harvard University Press, Cambridge, Mass., 1957. xxxi + 171 pp. 17 x 26 cm. Price $5.

The Neurohypophysis. Edited by H. HELLER. Academic Press, Inc., New York, 1957. xv + 275 pp. 19 x 25.5 cm. Price $9.50.

Expectant Motherhood. 3rd ed., revised. By NICHOLSON J. EASTMAN. Little, Brown & Company, Boston, 1957. xiv + 198 pp. 12.5 x 19 cm. Price $1.75.

Dorland's Illustrated Medical Dictionary. 23rd ed. W. B. Saunders Company, Philadelphia, 1957. xvii + 1,598 pp. 17 x 26 cm. Price $12.50.

The Adrenal Cortex. By I. CHESTER JONES. New York: Cambridge University Press, 1957. x + 315 pp. 14.5 x 22 cm. Price $7.00.

Technique of Organic Chemistry. Vol. X. Fundamentals of Chromatography. By HAROLD G. CASSIDY. Edited by Arnold Weissberger. Interscience Publishers, Inc., New York, 1957. xvii + 447 pp. 15 x 23 cm. Price $9.75.

The Chemistry of Organometallic Compounds. By EUGENE G. ROCHOW, DALLAS T. HURD, and RICHARD N. LEWIS. John Wiley & Sons, Inc., New York, 1957. vi + 344 pp. 15 x 23 cm. Price $8.50.

Dextran and Its Use in Colloidal Infusion Solutions. By ANDERS GRÖNWALL. Academic Press Inc., New York, 1957. 156 pp. 14 x 21.5 cm. Price $4.

Morphine & Allied Drugs. By A. K. REYNOLDS and LOWELL O. RANDALL. University of Toronto Press, Toronto, Canada, 1957. xiii + 393 pp. 15 x 23 cm. Price $10.

Clinical Toxicology of Commercial Products—Acute Poisoning (Home & Farm). By MARION N. GLEASON, ROBERT K. GOSSELIN, and HAROLD C. HODGE. The Williams & Wilkins Co., Baltimore, Md., 1957. xv + 1160 pp. 17 x 25.5 cm. Price $16.

A Guide to the Literature of Chemistry. 2nd ed. Edited by E. J. CRANE, AUSTIN M. PATTERSON, and ELEANOR B. MARR. John Wiley & Sons, Inc., New York, 1957. xiv + 395 pp. 15 x 23 cm. Price $9.50.

Drug Resistance of Microorganisms. By ROBERT J. SCHNITZER and EMANUEL GRUNBERG. Academic Press Inc., New York, 1957. xiv + 395 pp. 15 x 23 cm. Price $10.

Light, Vegetation and Chlorophyll. By J. TERRIEN, G. TRUFFAUT, and J. CARLES. Translated by Madge E. Thompson. Philosophical Library, Inc., New York, 1957. 228 pp. 12 x 18.5 cm. Price $6.

Methods of Biochemical Analysis. Vol. IV. Edited by DAVID GLICK. Interscience Publishers, Inc., New York, 1957. ix + 362 pp. 15 x 23 cm. Price $9.50.

British Pharmaceutical Codex 1954. (1957 Supplement). Edited by the Codex Revision Committee. The Pharmaceutical Press, London, 1957. xii + 124 pp. 14 x 22.5 cm. Price £1. 7. 6d.

The Structure of Nucleic Acids and Their Role in Protein Synthesis. Biochemical Society Symposia No. 14. Edited by E. M. CROOK. New York: Cambridge University Press, 1957. 74 pp. 16 x 25 cm. Price $3.75.

Book Notices

Hormones, Brain Function, and Behavior. Edited by HUDSON HOAGLAND. Academic Press, Inc., New York, 1957. 257 pp. 15 x 23.5 cm. Price $7.

This book is a report of a conference on neuroendocrinology held in May, 1956. Editor Hoagland notes that those particular metabolic messengers called hormones are formed in glands of internal secretion and released into the blood stream where they may be carried to all the body tissues before becoming inactive metabolites; and that relatively few of the hormone molecules reach target organs where they react with chemical receptors to act as regulating messengers affecting tissue and organ function. The twelve papers and discussions of the symposium attempt to elucidate selected aspects of hormone actions in relation to brain function and behavior. They are grouped under the following headings: effects of steroid hormones on the nervous system; sex hormones and behavior; serotonin, epinephrine, and their metabolites in relation to experimental psychiatry; the thryoid and behavior. The papers are documented and general author and subject indexes are appended. The book is a valuable review of the advanced thinking and working in this specialized field.

Clinical Physiology: The Functional Pathology of Disease. Edited by ARTHUR GROLLMAN. McGraw-Hill Book Company, Inc., New York, 1957. xvi + 854 pp. 16 x 23.5 cm. Price $12.50

This book is written by an outstanding worker in the fields of biochemistry, physiology, and pharmacology, and is augmented with the aid of twenty-five contributors. It presents to the senior medical student, the intern, and the medical practitioner the basic physiologic principles of clinical medicine. No attempt is made to cover the general subjects of physiology and clinical medicine, but emphasis is placed on those aspects of medical science which in the present stage of development have a practical application to the practice of medicine. The term "physiologic" is used in the spirit of Claude Bernard's definition as "the experimental basis of medicine," and this book offers a correlation of the basic disciplines: physiology, chemistry, pathology, and pharmacology. The text material is divided under the following general headings: General metabolic considerations, Cardiovascular system, Respiratory system, Digestive system, Hematopoietic system, Endocrine system, Renal excretory system, Locomotor system, Infection and immunity.

The style, format, and type are excellent. References are given at the end of each chapter, and a general index is appended. The book is a valuable contribution in its indicated field.

Modern Methods of Microscopy. (A Series of Papers Reprinted from "Research"). Edited by A. E. J. VICKERS. Interscience Publishers, Inc. New York, 1956. 114 pp. 18.5 x 25 cm. Price $3.50.

The papers collected in this cardboard backed book cover the following subjects: Modern microscopy,

Recent developments in electron microscopy, Development of reflecting microscopes, Applications of the reflecting microscope, Fluorescence microscopy, Numerical values and quantitative determinations, Surface microinterferometry, Metallurgical aspects of microscopy, Phase contrast microscopy, Microscopic interferometry, Flying spot microscope, X-ray microscopy, and Polarizing microscopy in organic chemistry and biology. The papers are illustrated and documented, and they should be useful to teachers and advanced workers in the field.

Collectanea Pharmaceutica Suecica. Edited by the Kungl. Pharmaceutiska Institutets Bibliotek, Stockholm. Franckellin Kirjapanio Ösakeyhtio, Helsinki, Finland, 1956. 56 pp. 15.5 x 23 cm.

This is a compilation of articles on subjects related to pharmacy that appeared in Scandinavian publications during 1955. English language articles are: A comparative study of different algesimetric methods in animals and man; Studies on antitubercular agents—syntheses of hydrazones of cyanoacetic acid hydrazide; Biochemical mutants of microorganisms in vitamin assay—assay of vitamin B_6; Studies on glycosides and iso-propylidene derivatives—synthesis by cation exchanger catalysis; The effect of vitamin K and naphthalene acetic acid on Datura; Sieves and powders in P. I. ed. 1; Recent advances in the chemistry of natural products from Latin America flora; The stability of acetyl-β-methylcholine iodide (Betacholyl) and succinylcholine iodide (Celocurin) in solution; The complex formation between iron (III) ion and some phenols. III. Salicylaldehyde, *o*-hydroxyacetophenone, salicylamide, and methyl salicylate. IV. The acidity constant of the phenolic group.

American Drug Index 1957. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Company, Philadelphia, Pa., 1956. 650 pp. 14 x 21 cm. Price $5.

This is the second edition of a comprehensive compilation of the names, composition, dosage forms, and indicated uses of pharmaceuticals and chemicals available to the medical and allied professions. The first edition was reviewed in THIS JOURNAL, **45**, 355(1956). The style and arrangement of the first edition are continued in the second edition, which contains additional entries and extend the book from 576 pages to 650 pages. A more serviceable binding has been used for the second edition. The thorough coverage of this constantly growing field makes the second edition and, we hope, future annual editions of this Index welcome.

Inhaltsstoffe und Prüfungsmethoden homöopathisch verwendeter Heilpflanzen. By HERBERT SCHINDLER. Editio Cantor, Aulendorf i. Württ., 1955. 231 pp. 21 x 30 cm.

The cited work represents a corrected and complete indexed reprint of a series of articles which appeared in the columns of the outstanding German periodical *Arzneimittel-Forschung*. With the exception of about four pages, the text is monographic, treating in considerable detail a total, of 115 crude drugs in alphabetic array, many of which are important in American practice, others of primary importance in the old world (such as *Viscum album*, *Menyanthes*). The content is of value on account of original contributions from the laboratory of Dr. Willmar Schwabe G.m.b.H. (Karlsruhe) and also because of the comprehensive review of the literature which has been scrupulously studied and brought up almost to the current year. For instance, Rhubarb root is covered in four 2-columned pages under the following headings: originating plant, range, parts used, preparation of homeopathic medicinals, usage, constituents, testing (the last two have the greatest emphasis with considerable detail on chromatography and absorption spectrography); there are 116 literature references, some of these as recent as 1954. With the exception of the minor treatment of homeopathic preparations which is of such little importance to us now, this volume is a rich treasure of useful and important information. Obviously, great care was paid to accuracy and thoroughness of coverage.

The Encyclopedia of Chemistry. Edited by GEORGE L. CLARK and GESSNER G. HAWLEY. Reinhold Publishing Corporation, New York, 1957. xvi + 1,037 pp. 18 x 26 cm. Price $19.50.

This book is described as a one-volume encyclopedia centered around the subject of chemistry and including over 20 different sciences that border on chemistry. This extremely wide coverage in slightly over one thousand pages demands judicious selectivity and condensation. This task has been assigned to over 500 contributors. An outstanding example of the effort made to obtain the best men for the task is the 5-page essay on chemical literature by E. J. Crane, editor of *Chemical Abstracts*. Within the spatial limitations, the subjects from Abrasion Resistance to Zsigmondy, Richard, are presented in an interesting manner. The many fields included in the compilation might lead one to consider this a "book of technical knowledge" and its place in a reference library would probably be directed by its consideration as such.

The book itself is a splendid physical product with clear and convenient style and format and an attractive, strong binding that permits pages to remain flat wherever the book is opened. The inclusion of a general index serves as a cross index to the alphabetically arranged text and as a useful guide to subjects that are included in discussions under other general headings. The book should be a useful addition to any reference library.

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